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SYMBOLIC PROOF OF BISTABILITY IN REACTION NETWORKS

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ABSTRACT. Deciding whether and where a system of parametrized ordinary differential equations displays bistability, that is, has at least two asymptotically stable steady states for some choice of parameters, is a hard problem. For systems modeling biochemical reaction networks, we introduce a procedure to determine, exclusively via symbolic computations, the stability of the steady states for unspecified parameter values. In particular, our approach fully determines the stability type of all steady states of a broad class of networks. To this end, we combine the Hurwitz criterion, reduction of the steady state equations to one univariate equation, and structural reductions of the reaction network. Using our method, we prove that bistability occurs in open regions in parameter space for many relevant motifs in cell signaling.

1. INTRODUCTION

Bistability, that is, the existence of at least two stable steady states in a dynamical system, has been linked to switch-like behavior in biological networks and cellular decision making and it has been observed experimentally in a variety of systems [37, 47, 48]. However, proving the existence of bistability in a parameter-dependent mathematical model is in general hard.

We focus on (bio)chemical reaction networks with associated kinetics, giving rise to systems of Ordinary Differential Equations (ODEs) that model the change in the concentration of the species of the network over time. These systems come equipped with unknown parameters and, ideally, one wishes to determine properties of the family of ODEs for varying parameter values. Here we are concerned with stability of the steady states, and focus on the following three questions: (1) if the network admits only one steady state for all parameter choices, is it asymptotically stable? (2) can parameter values be chosen such that the system is bistable? (3) does it hold that for any choice of parameters yielding at least three steady states, two of them are asymptotically stable?

As the parameters are regarded unknown, explicit expressions for the steady states are rarely available. Problem (1) has been shown to be tractable for certain classes of networks. For example, the only positive steady state of complex balanced networks admits a Lyapunov function making it asymptotically stable [28, 40]. The use of Lyapunov functions and the theory of monotone systems has been employed more broadly to other classes of networks [1, 3, 23]. Finally, algebraic criteria as the Hurwitz criterion or the study of P-matrices also provide asymptotic stability of steady states, often in combination with algebraic parametrizations of the steady state variety. In [13] the Hurwitz criterion is analyzed using graphical methods.

Problem (2) is much harder and typically tackled by first deciding whether the network admits multiple steady states using one of the many available methods [15, 26, 43, 45], and then numerically computing the steady states and their stability for a suitable choice of parameter values. Rigorous proofs of bistability require advanced analytical arguments such as bifurcation theory and geometric singular perturbation theory, as employed in [31, 59] for futile cycles. The use of the Hurwitz criterion to prove bistability is anecdotal, as rarely explicit descriptions of the steady states can be found. Problem (3) has been addressed for small systems using case-by-case approaches, but no systematic strategy has been proposed.

We devise a flow chart to solve the problems (1)-(3) using computer-based proofs relying only on symbolic operations. This is achieved by combining three key ingredients. First, we apply

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the Hurwitz criterion \[2, 6\] on the characteristic polynomial of the Jacobian of the ODE system evaluated at a parametrization of the steady states. Second, we observe that when all but the last Hurwitz determinants are positive (meaning that instabilities only arise via an eigenvalue with positive real part), and further the solutions to the steady state equations are in one to one correspondence with the zeros of a univariate function, then the stability of the steady states is completely determined and question (3) can be answered (Theorem 1). Third, if Theorem 1 does not apply, whenever possible, we reduce the network to a smaller one for which Theorem 1 applies. Afterwards, we use the results on the reduced network to infer stability properties of the steady states of the original network in an open parameter region. To this end, many reduction techniques have been proposed \[5, 10, 20, 34, 42\], but often removal of reactions \[42\] and of intermediates \[34\] suffice for (bio)chemical networks.

Even though our approach demands heavy symbolic computations, we illustrate how problem (3) can successfully be tackled for small networks, and further, we prove the existence of bistability in open regions of the parameter space for several relevant cell signaling motifs. In particular, for many networks we provide the first proof of bistability in an open region of the parameter space, with the exception of the double phosphorylation cycle, whose bistability was proven in \[39\].

This article is structured as follows: In Section 2, we provide the mathematical framework and state the two main results of this work. In Section 3 we present the procedure for detecting bistability and apply it to numerous examples arising from cell signalling. In Section 4 we prove the main results, and in Section 5 we address the computational challenges of our method. Finally, we conclude with a discussion of our contribution. We include an appendix with an expanded explanation of the examples in Section 3 together with additional stability criteria that can be considered instead of the Hurwitz criterion. Computations are provided in an accompanying supplementary Maple file.

2. Mathematical framework

We use the following notation: \(J_f\) is the Jacobian matrix of a function \(f\). We denote by \(V^\perp\) the orthogonal complement of a vector space \(V\). For \(A \in \mathbb{R}^{n \times n}\) and \(I, J \subseteq \{1, \ldots, n\}\), we let \(A_{I,J}\) be the submatrix of \(A\) with rows (resp. columns) with indices in \(I\) (resp. \(J\)). Finally, we denote by \(p_A(\lambda)\) the characteristic polynomial of a matrix \(A\).

2.1. Reaction networks and steady states. We consider reaction networks over a set of species \(S = \{X_1, \ldots, X_n\}\) given by a collection of reactions

\[
(1) \quad r_j: \sum_{i=1}^n \alpha_{ij} X_i \longrightarrow \sum_{i=1}^n \beta_{ij} X_i \quad \text{for } j = 1, \ldots, m
\]

with \(\alpha_{ij} \neq \beta_{ij}\) for at least one index \(i\). Let \(x_i\) denote the concentration of \(X_i\). Given a differentiable kinetics \(v: \mathbb{R}_{\geq 0}^n \to \mathbb{R}_{\geq 0}^n\), the dynamics of the concentrations of the species in the network over time \(t\) are modeled by means of a system of autonomous ODEs,

\[
(2) \quad \frac{dx}{dt} = Nx(x), \quad x = (x_1, \ldots, x_n) \in \mathbb{R}_{\geq 0}^n,
\]

where \(N \in \mathbb{R}^{n \times m}\) is the stoichiometric matrix with \(j\)th column \((\beta_{1j} - \alpha_{1j}, \ldots, \beta_{nj} - \alpha_{nj})\). That is, the \(j\)-th column of \(N\) encodes the net production of each species in the \(j\)-th reaction. We write

\[
 f(x) := Nx(x).
\]

With mass-action kinetics, we have \(r_j(x) = \kappa_j x_1^{a_{1j}} \cdots x_n^{a_{nj}}\) where \(\kappa_j > 0\) is a rate constant, shown often as a label of the reaction.

Under mild conditions, satisfied by common kinetics including mass-action, \(\mathbb{R}_{\geq 0}^n\) and \(\mathbb{R}_{\geq 0}^n\) are forward invariant by Eq. (2). Furthermore, any trajectory of Eq. (2) is confined to a so-called stoichiometric compatibility class \((x_0 + S) \cap \mathbb{R}_{\geq 0}^n\) with \(x_0 \in \mathbb{R}_{\geq 0}^n\), where \(S\) is the column span of \(N\) and is called the stoichiometric subspace \[29\]. We let \(s = \text{dim}(S)\) and \(d = n - s\). The set \((x_0 + S) \cap \mathbb{R}_{\geq 0}^n\) is the solution set of the equations \(Wx = T\) with \(W \in \mathbb{R}^{d \times n}\) any matrix whose rows form a basis of \(S^\perp\) and \(T = Wx_0 \in \mathbb{R}^d\). These equations are called conservation
laws and the defined stoichiometric compatibility class is denoted by $P_T$. The vector $T$ is called a vector of total amounts.

The steady states (or equilibria) of the network are the non-negative solutions to the system $N v(x) = 0$. The positive steady states, that is, the solutions in $\mathbb{R}_{>0}^d$, define the positive steady state variety $V^+$. The existence of $d$ linearly independent conservation laws implies that $d$ steady state equations are redundant. Let $W \in \mathbb{R}^{d\times n}$ be row reduced with $i_1, \ldots, i_d$ the indices of the first non-zero coordinate of each row. For $T \in \mathbb{R}^d$, we define the following function

$$F_T(x) = \begin{cases} f_i(x) & i \notin \{i_1, \ldots, i_d\} \\ (W x - T)_i & i \in \{i_1, \ldots, i_d\}, \end{cases}$$

which arises after replacing redundant equations in the system $N v(x) = 0$ with $W x - T = 0$. Hence, the solutions to $F_T(x) = 0$ are the steady states in the stoichiometric compatibility class $P_T$ [14, 57]. A steady state $x^*$ is said to be non-degenerate if $\ker(J_f(x^*)) \cap S = \{0\}$, or equivalently, if $\det(J_{F_{x^*}}(x^*)) \neq 0$ [57]. Observe that $J_{F_{x^*}}(x^*)$ is independent of $T$.

**Example 1.** Consider the following reaction network

$$X_1 \xrightarrow{\kappa_1} X_2 \quad X_2 + X_3 \xrightarrow{\kappa_2} X_1 + X_4 \quad X_4 \xrightarrow{\kappa_3} X_3.$$  

This is a simplified model of a two-component system, consisting of a histidine kinase HK and a response regulator RR [14]. Both occur unphosphorylated $(X_1, X_3)$ and phosphorylated $(X_2, X_4)$. With mass-action kinetics, the associated ODE system is

$$\frac{dx_1}{dt} = -\kappa_1 x_1 + \kappa_2 x_2 x_3 \quad \frac{dx_2}{dt} = \kappa_1 x_1 - \kappa_2 x_2 x_3 \quad \frac{dx_3}{dt} = -\kappa_2 x_2 x_3 + \kappa_3 x_4 \quad \frac{dx_4}{dt} = \kappa_2 x_2 x_3 - \kappa_3 x_4,$$

and we consider the stoichiometric compatibility class $P_T$ defined by $x_1 + x_2 = T_1$ and $x_3 + x_4 = T_2$. With this choice, $F_T(x)$ is

$$(x_1 + x_2 - T_1, \kappa_1 x_1 - \kappa_2 x_2 x_3, x_3 + x_4 - T_2, \kappa_2 x_2 x_3 - \kappa_3 x_4)^{tr},$$

where $tr$ indicates the transpose.

In this example, the positive steady states are the positive solutions to the equations $\kappa_1 x_1 - \kappa_2 x_2 x_3 = 0$, $\kappa_2 x_2 x_3 - \kappa_3 x_4 = 0$, obtained by setting the right-hand side of $\frac{dx_1}{dt}$, $\frac{dx_2}{dt}$ in Eq. (6) to zero. Solving these equations for $x_1$ and $x_3$ leads to the following parametrization of $V^+$:

$$\phi(x_2, x_4) = \left( \frac{\kappa_4}{\kappa_1} x_2, \frac{\kappa_4}{\kappa_2} x_2, x_4 \right), \quad (x_2, x_4) \in \mathbb{R}_{>0}^2.$$

In general, we refer to a positive parametrization as any surjective map of the form

$$\phi: \mathbb{R}_{>0}^d \rightarrow V^+ \quad \xi \mapsto \phi(\xi).$$

In practice, under mass-action kinetics, the entries of $\phi$ are often rational functions in $\xi$. Positive parametrizations play a key role in what follows, and strategies to find one have been briefly reviewed in [14]. Note that there is no guarantee that a positive parametrization exists, or that can be found with known approaches. Nevertheless, networks arising from cell signaling with mass-action kinetics display features that facilitate the existence of parametrizations. The main such property is that monomials in $v(x)$ are often of at most degree two. This allows to perform linear elimination and write some variables in terms of positive functions of the others at steady state. If enough variables can be eliminated, then a positive parametrization can be found. This approach has been extensively studied, e.g. [33, 35, 38, 54, 55]. The second scenario that favors finding parametrizations is when the positive steady state variety is described by binomial equations. In this case, $V^+$ is parametrized by monomials. Complex-balancing belongs to this scenario [19, 22, 28, 40, 56], as well as so-called networks with toric steady states [51]. Simple ways to find parametrizations for certain classes of networks (MESSI networks) have been also identified [50]. Finally, it is worth mentioning that often, brute force with symbolic software (such as Maple) works: try to solve symbolically the steady state equations for subsets of $s$ variables. If an output is produced, then decide whether/when the solution is positive.
2.2. Multistationarity, bistability and network reduction. We say that a network is multistationary if it has at least two positive steady states in some \( \mathcal{P}_T \), that is, \( F_T(x) = 0 \) has at least two positive solutions for some \( T \in \mathbb{R}^d \). Similarly, a monostationary network has exactly one positive steady state in each \( \mathcal{P}_T \).

Under some conditions, if the sign of \( \text{det}(J_{F_T}(x^*)) \) is \((-1)^s \) for all positive steady states \( x^* \), then the network is known to be monostationary; if the sign is \((-1)^{s+1} \) for some \( x^* \), then it is multistationary [14, 16]. We refer to [14] for a positive parametrization of \( V^+ \) available, this result can be used to obtain inequalities in the rate constants that guarantee or preclude multistationarity [14, 16].

Given an ODE system \( \frac{dx}{dt} = f(x) \), a steady state \( x^* \) is said to be stable if for each \( \epsilon > 0 \), there exists \( \delta > 0 \) such that solutions starting within distance \( \delta \) of \( x^* \), remain within distance \( \epsilon \) of \( x^* \). If additionally \( \delta \) can be chosen such that solutions tend to \( x^* \) as time increases, then \( x^* \) is asymptotically stable. If \( x^* \) is not stable, then it is unstable.

Stability of steady states can be often addressed by inspecting the eigenvalues of \( J_f(x^*) \): if all eigenvalues of \( J_f(x^*) \) have negative real part, then \( x^* \) is said to be exponentially stable, and exponential stability implies asymptotic stability (§2.7-2.8 in [52]). If at least one eigenvalue has positive real part, then \( x^* \) is unstable.

The stability of a steady state is studied relatively to \( \mathcal{P}_T \). A network that admits at least two asymptotically stable positive steady states in some \( \mathcal{P}_T \) is called bistable. Detecting multistationarity and bistability is challenging already for medium sized networks. To overcome computational difficulties one may employ several structural modifications of the network, among which we focus on removal of reactions (subnetworks) and removal of intermediates.

Specifically, given two networks \( G \) and \( G' \), \( G' \) is a subnetwork of \( G \) if it arises after removing reactions of \( G \) [42]. Assuming mass-action kinetics and assuming that \( G \) and \( G' \) have the same stoichiometric subspace, if \( G' \) has \( \ell_1 \) exponentially stable and \( \ell_2 \) non-degenerate unstable steady states in some stoichiometric compatibility class for some rate constants \( \kappa \), then \( G \) has at least \( \ell_1 \) exponentially stable and \( \ell_2 \) non-degenerate unstable steady states in some stoichiometric compatibility class for some rate constants \( \tilde{\kappa} \). Here, \( \tilde{\kappa} \) agrees with \( \kappa \) for the common reactions and is small enough for the reactions that only are in \( G \).

An intermediate \( Y \) is any species that is both a product and a reactant in the network and that does not interact with any other species, that is, the only complex containing it is \( Y \) itself. Removal of intermediates leads to a new network obtained after collapsing into one reaction all paths of reactions from to and from non-intermediates and through intermediates [34]. For example, the species \( S_0E \) in

\[
S_0 + E \iff S_0E \longrightarrow S_1 + E
\]

is an intermediate. Its removal yields the reaction \( S_0 + E \longrightarrow S_1 + E \). This is one of the simplest forms of intermediates, but these can appear in complicated mechanisms linking several non-intermediate complexes.

With mass-action kinetics, if \( G' \) is obtained from \( G \) through the removal of intermediate species, and satisfies a technical condition on the rate constants [34] [53], the following holds: If \( G' \) has \( \ell_1 \) exponentially stable and \( \ell_2 \) non-degenerate unstable steady states in some stoichiometric compatibility class for some rate constants \( \kappa \), then \( G \) has at least \( \ell_1 \) exponentially stable and \( \ell_2 \) non-degenerate unstable steady states in some stoichiometric compatibility class for some rate constants \( \tilde{\kappa} \). In [34] [53], an explicit description of the rate constants \( \tilde{\kappa} \) in terms of \( \kappa \) can be found. The technical condition on the rate constants is satisfied by intermediates \( Y \) appearing only in subnetworks of the form \( y \longrightarrow Y \longrightarrow y' \) or \( y \iff Y \longrightarrow y' \), where \( y, y' \) are arbitrary complexes, see [53] Prop. 5.3(i)]. This is the main type of intermediates arising in cell signaling, and in particular in this work, and hence bistability and multistationarity can be lifted.

There are other network reduction techniques that yield to analogous results, see e.g. [5] [10]. In what follows, we will only focus on removal of reactions and intermediates, as these modification typically suffice in networks arising from cell signaling.

2.3. The Jacobian matrix of reaction networks. In the context of reaction networks, we determine the stability of steady states based on the eigenvalues of the Jacobian of the restriction of the system in Eq. (2) to a stoichiometric compatibility class. To this end, we consider the
Furthermore, for a positive steady state \( x (10) \) to every \( z \in \phi \)

**Proposition 2.** Let \( J \) be a matrix whose columns form a basis of \( S \), and \( L \in \mathbb{R}^{s \times m} \) be such that \( N = R_0 L \). Then the projection of \( J_f(x) \) onto \( S \) is \( LJ_v(x)R_0 \).

For the network in Eq. (4), we consider the following matrices \( Q \)

Further, the independent term of \( q_x(\lambda) \) is \((-1)^s \det(J_{F_T}(x))\), with \( F_T \) as in Eq. (3) for any choice of \( W \).

The proof of Proposition 1 can be found in Section 4 as part of Proposition 3. According to Proposition 2, the eigenvalues of the matrix \( Q_x(\lambda) \) are the eigenvalues of \( J_f(x) \) once zero counted with multiplicity \( d \) is disregarded. In order to study the (sign of the real part of the) spectrum of the matrices \( Q_x \) when \( x \) is a positive steady state, we use a positive parametrization.

**Example 2.** For the network in Eq. (4), we consider the following matrices

\[
R_0 = \begin{bmatrix} -1 & 0 \\ 1 & 0 \\ 0 & -1 \end{bmatrix}, \quad L = \begin{bmatrix} 1 & -1 & 0 \\ 0 & -1 & 1 \end{bmatrix}, \quad J_v(x) = \begin{bmatrix} \kappa_1 & 0 & 0 & 0 \\ 0 & \kappa_2 x_3 & \kappa_2 x_2 & 0 \\ 0 & 0 & 0 & \kappa_3 \end{bmatrix},
\]

and we are interested in the eigenvalues of the matrix

\[
(9) \quad Q_x = L J_v(x) R_0 = \begin{bmatrix} -\kappa_1 - \kappa_2 x_3 & -\kappa_2 x_2 \\ -\kappa_2 x_3 & -\kappa_2 x_2 - \kappa_3 \end{bmatrix}
\]

evaluated at a steady state \( x^* = \phi(x_2, x_4) \), c.f. Eq. (7). Thus, by analyzing the eigenvalues of \( Q_{\phi(x_2,x_4)} \) for all values of \( \kappa \) and \( x_2, x_4 > 0 \), we study the stability of all positive steady states. In particular, the characteristic polynomial \( q_x(\lambda) \) of \( Q_x \) in Eq. (9) is

\[
q_x(\lambda) = \lambda^2 + (\kappa_2 x_2 + \kappa_2 x_3 + \kappa_1 + \kappa_3) \lambda + \kappa_1 \kappa_2 x_2 + 2 \kappa_2 \kappa_3 x_3 + \kappa_1 \kappa_3.
\]

We conclude this part with a key technical result (proven in Section 3) on the determinant of \( J_f(x^*) \) in the particular case where system \( F_T(x) = 0 \) is reduced to one univariate equation.

**Proposition 2.** Let \( W \in \mathbb{R}^{d \times n} \) be a row reduced matrix whose rows form a basis of \( S^\perp \), and let \( T \in \mathbb{R}^d \) be fixed. Consider \( F_T \) as in Eq. (3). Assume that there exist an open interval \( \mathcal{E} \subseteq \mathbb{R} \), a differentiable function \( \varphi : \mathcal{E} \to \mathbb{R}_{>0} \), and an index \( j \) such that \( F_T(\varphi(z)) = 0 \) for all \( \ell \neq j \) and every \( z \in \mathcal{E} \). Then, the set of positive solutions of the system \( F_T(x) = 0 \) contains the solutions to

\[
(10) \quad F_{T,J}(\varphi(z)) = 0, \quad x_\ell = \varphi_\ell(z), \quad \ell = 1, \ldots, n \text{ and } z \in \mathcal{E}.
\]

Furthermore, for a positive steady state \( x^* = \varphi(z) \) such that \( \varphi_i'(z) \neq 0 \) for some \( i \), it holds

\[
\det(J_{F_T}(x^*)) = \frac{(-1)^{i+j}}{\varphi_i'(z)} (F_{T,J} \circ \varphi)'(z) \det(J_{F_T}(x^*), I, J),
\]

where \( I = \{1, \ldots, n\} \setminus \{i\} \) and \( J = \{1, \ldots, n\} \setminus \{j\} \).

In practice, \( \varphi_i(z) = z, \mathcal{E} \subseteq \mathbb{R}_{>0} \), and the solutions to \( F_T(x) = 0 \) are in one to one correspondence with the solutions to Eq. (10). In this case, given the positive solutions \( z_1 < \cdots < z_\ell \) of Eq. (10), the sign of the derivative of \( F_{T,J}(\varphi(z)) \) evaluated at \( z_1, \ldots, z_\ell \) alternates if all the steady states are non-degenerate. If additionally the sign of \( \frac{1}{\varphi(z)} \det(J_{F_T}(\varphi(z)), I, J) \) is independent of the choice of \( z \), then the sign of \( \det(J_{F_T}(\varphi(z))) \) depends only on the sign of the derivative of \( (F_{T,J} \circ \varphi) \) at \( z_\ell \) and hence alternates as well. We will exploit this fact below.
2.4. **Algebraic criteria for (bi)stability.** We present now the Hurwitz criterion [2, 6], which is used to decide whether all the roots of a polynomial have negative real part, or whether there is at least one root with positive real part.

**Criterion 1 (Hurwitz).** Let \( p(x) = a_2x^2 + \ldots + a_0 \) be a real polynomial with \( a_2 > 0 \) and \( a_0 \neq 0 \). The Hurwitz matrix \( H = (h_{ij}) \) associated with \( p \) has entries \( h_{ij} = a_{s-2i+j} \) for \( i, j = 1, \ldots, s \), by letting \( a_k = 0 \) if \( k \notin \{0, \ldots, s\} \):

\[
H = \begin{bmatrix}
a_{s-1} & a_s & 0 & 0 & \cdots & 0 \\
a_{s-3} & a_{s-2} & a_{s-1} & 0 & \cdots & 0 \\
0 & 0 & 0 & a_{6-s} & \cdots & a_2 \\
0 & 0 & 0 & 0 & \cdots & a_0 \\
\end{bmatrix} \in \mathbb{R}^{s \times s}.
\]

The \( i^{th} \) Hurwitz determinant is defined to be \( H_i = \det(H_{i,i}) \), where \( I = \{1, \ldots, i\} \). Then, all roots of \( p \) have negative real part if and only if \( H_i > 0 \) for all \( i = 1, \ldots, s \). If \( H_i < 0 \) for some \( i \), then at least one root of \( p \) has positive real part.

Importantly, \( H_s = a_0 H_{s-1} \). Pairs of imaginary roots (leading to Hopf bifurcations) arise when at least one of the \( H_i \) vanish [58] (see [17] in the context of reaction networks).

For the polynomial \( q_x(\lambda) \) or \( q_{\varphi(z)}(\lambda) \), the Hurwitz determinants are typically rational functions in \( x \) or \( z \).

**Example 3.** In Example 2 the Hurwitz determinants of the characteristic polynomial \( q_x \) are

\[
H_1 = \kappa_2(x_2 + x_3) + \kappa_1 + \kappa_3, \quad \text{and} \quad H_2 = (\kappa_1x_2 + \kappa_3x_3)\kappa_2 + \kappa_1\kappa_3)H_1.
\]

Both determinants are polynomials in \( \kappa, x \) with positive coefficients and hence positive for all \( \kappa \in \mathbb{R}^3 \) and \( x \in \mathbb{R}^2_{>0} \). By the Hurwitz criterion, any positive steady state is exponentially stable. This network has exactly one steady state in each stoichiometric compatibility class [14], and we now additionally conclude that the only steady state is exponentially stable.

Often for small networks, all but the last Hurwitz determinants are positive. Then, the stability of a steady state \( x^* \) is fully determined by the sign of \( H_s \), which agrees with the sign of the independent term of \( q_{x^*}(\lambda) \), which in turn is \((-1)^s \det(J_{F_T}(x^*))\) by Proposition 1. Together with Proposition 2 we obtain the following theorem, proved in Section 4.

**Theorem 1.** Let \( T, \mathcal{E}, \varphi, i, j, I, J \) be as in Proposition 2 and \( q_{x^*} \) be the characteristic polynomial of the matrix \( Q_{x^*} \) as in Proposition 4. Assume that

- the sign of \( \frac{\varphi'(z)}{\varphi(z)} \det(J_{F_T}(\varphi(z)))_{I,I} \) is independent of \( z \in \mathcal{E} \) and is nonzero, and
- the first \( s - 1 \) Hurwitz determinants of \( q_{x^*} \) are positive for all positive steady states \( x^* \).

If \( z_1 < \cdots < z_\ell \) are the positive solutions to Eq. (10) and all are simple, then either \( \varphi(z_1), \varphi(z_2), \ldots \) are exponentially stable and \( \varphi(z_2), \varphi(z_3), \ldots \) are unstable, or the other way around. Specifically, \( \varphi(z_1) \) is exponentially stable if and only if

\[
(-1)^{s+i+j} \frac{\varphi'(z_1)}{\varphi(z_1)}(F_{T,j} \circ \varphi)'(z_1) \det(J_{F_T}(\varphi(z_1)))_{J,J} > 0.
\]

In practice, \( F_{T,j}(\varphi(z)) = \frac{\varphi(z)}{\varphi'(z)} b(z) \) is a rational function in \( z \) with \( b(z) > 0 \) in \( \mathcal{E} \). Then the zeros of \( F_{T,j}(\varphi(z)) \) are the roots of \( a(z) \), and the signs of \( (F_{T,j} \circ \varphi)'(z^*) \) and \( a'(z^*) \) agree for all \( z^* \in \mathcal{E} \) such that \( a(z^*) = 0 \) (Lemma 1 in Section 4).

**Example 4.** We illustrate Theorem 1 with a hybrid histidine kinase network with mass-action kinetics [14], see Fig. 1. We rename the species as follows: \( X_1 = \text{HK}_{\text{up}} \), \( X_2 = \text{HK}_{\text{pd}} \), \( X_3 = \text{HK}_{\text{pp}} \), \( X_4 = \text{HK}_{\text{pp}} \), \( X_5 = \text{Htp} \) and \( X_6 = \text{Htp}_{\text{pp}} \). The associated ODE system is

\[
\begin{align*}
\frac{dx_1}{dt} &= -\kappa_1 x_1 + \kappa_4 x_3 x_5 \\
\frac{dx_2}{dt} &= \kappa_1 x_1 - \kappa_2 x_2 + \kappa_5 x_4 x_5 \\
\frac{dx_3}{dt} &= -\kappa_3 x_3 + \kappa_2 x_2 - \kappa_4 x_3 x_5 \\
\frac{dx_4}{dt} &= \kappa_3 x_3 - \kappa_5 x_4 x_5 \\
\frac{dx_5}{dt} &= -\kappa_4 x_3 x_5 - \kappa_5 x_4 x_5 + \kappa_6 x_6 \\
\frac{dx_6}{dt} &= \kappa_4 x_3 x_5 - \kappa_6 x_6 + \kappa_5 x_4 x_5.
\end{align*}
\]
Hybrid histidine kinase
HK\textsubscript{00} → HK\textsubscript{p0} → HK\textsubscript{pp} → Htp
HK\textsubscript{pp} + Htp → HK\textsubscript{p0} + Htp
HK\textsubscript{p0} + Htp → HK\textsubscript{00} + Htp

Two substrate enzyme catalysis
E + S\textsubscript{1} ⇔ ES\textsubscript{1} E + S\textsubscript{2} ⇔ ES\textsubscript{2} ES\textsubscript{1}S\textsubscript{2} ⇔ E + P
S\textsubscript{2} + ES\textsubscript{1} ⇔ ES\textsubscript{1}S\textsubscript{2} S\textsubscript{1} + ES\textsubscript{2} ⇔ ES\textsubscript{1}S\textsubscript{2}

Gene transcription network
X\textsubscript{1} → X\textsubscript{1} + P\textsubscript{1} X\textsubscript{2} → X\textsubscript{2} + P\textsubscript{2} P\textsubscript{1} → 0 P\textsubscript{2} → 0
X\textsubscript{2} + P\textsubscript{1} ⇔ X\textsubscript{2}P\textsubscript{1} 2P\textsubscript{2} ⇔ P\textsubscript{2}P\textsubscript{2} X\textsubscript{1} + P\textsubscript{2}P\textsubscript{2} ⇔ X\textsubscript{1}P\textsubscript{2}P\textsubscript{2}

Figure 1. Three networks where stability of steady states is fully determined.

The conservation laws of the system are $T_1 = x_1 + x_2 + x_3 + x_4$, $T_2 = x_5 + x_6$. Hence, by Eq. (5),

$$F_T(x) = \begin{bmatrix}
  x_1 + x_2 + x_3 + x_4 - T_1 \\
  \kappa_5 x_4 x_5 + \kappa_1 x_1 - \kappa_2 x_2 \\
  -\kappa_4 x_3 x_5 + \kappa_2 x_2 - \kappa_3 x_3 \\
  -\kappa_5 x_4 x_5 + \kappa_3 x_3 \\
  x_3 + x_6 - T_2 \\
  \kappa_4 x_3 x_5 + \kappa_5 x_4 x_5 - \kappa_6 x_6
\end{bmatrix}.$$  

Here $s = 4$. The existence of three positive steady states for this network was established in [14]. We compute $q_x$ and the Hurwitz determinants in Maple 2019 (see accompanying Maple file) and obtain that all but the last are polynomials in $x$ and $\kappa$ with positive coefficients; hence they are positive when evaluated at a positive steady state.

We proceed to decide whether Theorem 1 applies. In [14], it was shown that the assumptions of Proposition 2 hold with $i = j = 5$, with $z = x_5$, $F_{T,5}$ corresponding to the conservation law with $T_2$, and $\epsilon = \mathbb{R}_{>0}$. That is, the solutions to the four steady state equations together with the conservation law associated with $T_1$ can be parametrized by a function $\varphi$ that only depends on $z = x_5$. The denominator of $(F_{T,5} \circ \varphi)(z)$ is positive and its numerator is a polynomial of degree 3 in $z$, which can have 1, 2 or 3 positive roots, depending on the choice of parameters. Additionally, $\det(J_{F_T}(\varphi(x_5)),_{1,1})$ is a rational function with all coefficients positive. Thus, we are in the situation of Theorem 1. The independent term of the numerator of $(F_{T,5} \circ \varphi)(z)$ is negative, its first root has positive derivative. Furthermore, the sign of $\frac{(-1)^{s+i+j}}{\varphi'(z_1)}(F_{T,j} \circ \varphi)'(z_1) \det(J_{F_T}(\varphi(z_1)),_{1,1})$ is positive as well. We conclude, using Theorem 1 that whenever the network has three positive steady states coming from the roots $z_1 < z_2 < z_3$ of $(F_{T,5} \circ \varphi)(z)$, then the steady states $\varphi(z_1)$ and $\varphi(z_3)$ are exponentially stable and the steady state $\varphi(z_2)$ is unstable. We have shown that

this network displays bistability whenever there are three positive steady states.

Theorem 1 implies the existence of bistability if the network admits at least three positive steady states and (11) holds. Indeed, as the stability of the positive steady states alternates and that the first one is exponentially stable (when ordered as in Theorem 1), there exist at least two exponentially stable steady states. If (11) does not hold, then bistability will only follow if the network admits four positive steady states.

If $F_{T,j}(\varphi(z))$ is a rational function with positive denominator, we verify (11) by finding the sign of $\frac{1}{\varphi'(z)} \det(J_{F_T}(\varphi(z)),_{1,1})$ (which is independent of $z$), and inspecting the sign of the independent term of the numerator of $F_{T,j}(\varphi(z))$ to deduce the sign of $(F_{T,j} \circ \varphi)'(z_1)$. 


In order to establish that the network admits three positive steady states, several strategies can be employed. First, one can directly attempt to find values of the parameters for which the numerator of $F_{T,j}(\varphi(z))$ has three positive roots. By Descartes’ rule of signs and the degree of the polynomial, upper bounds on the possible number of positive roots can easily be found.

Alternatively, numerous existing methods to determine multistationarity [14, 15, 24, 26, 45, 51] can be employed, see [43] for a review. Most of these methods give a choice of (rational) parameters for which the network has at least two positive steady states, but we require three. An exception is the method in [8], which via the study of the Newton polytope of the system of interest, might directly certify the existence of at least three positive solutions.

For the purpose of finding three positive steady states, perhaps the simplest approach is to consider a choice of parameters for which multistationarity exists, as for example returned by the CRNT toolbox [26], and find the solutions to the corresponding system $F_T(x) = 0$ (or equivalently $F_{T,j}(\varphi(z)) = 0$) using available software. This will typically provide the three desired solutions. Note that this last step is not necessarily symbolic. To certify that chosen parameters give rise to three positive steady states using symbolical methods, we use Sturm sequences on the numerator of $F_{T,j}(\varphi(z))$ to count the number of positive roots [7].

In this work, we have mainly applied the method in [14] to determine multistationarity. The approach works for mass-action kinetics and relies on the determinant of $J_{F_T}(\phi(\xi))$, for a positive parametrization $\phi$. Assume the network is conservative and has no steady state at the boundary of any $P_r$. Then, if $\kappa$ and $\xi$ are such that $(-1)^\kappa \det(J_{F_T}(\phi(\xi))) < 0$, then the network displays multistationarity in the stoichiometric compatibility class containing $\phi(\xi)$.

3. Symbolic determination of stability

We now combine the ingredients introduced in the previous section into a strategy to determine the stability of positive steady states and, importantly, detect bistability, using (mainly) the Hurwitz criterion and Theorem 1. Given a reaction network with kinetics $v(x)$ the steps taken are depicted in Fig. 2. Specifically, we find the characteristic polynomial $q_x$ and the Hurwitz determinants. If all determinants are positive, then all positive steady states are exponentially stable. If only the last Hurwitz determinant can be negative, then we attempt to apply Theorem 1. We only find a parametrization $\phi$ as in Eq. (8) when the sign of $H_i$ is not

![Figure 2](image-url). Flow chart of the proposed approach to study the stability of steady states. $H_i$ denotes the $i$-th Hurwitz determinant of $q_x$. To decide whether $H_i(x^*) > 0$ for all $x^* \in V^+$ and some $i$, first check whether $H_i(x) > 0$ for all $x \in \mathbb{R}^{s}_{>0}$. If not, find a positive parametrization $\phi$ (if possible, see text), and decide whether $H_i(\phi(\xi)) > 0$ for all $\xi \in \mathbb{R}^{d}_{>0}$. If a parametrization cannot be found, or the statement $H_i(x^*) > 0$ for all $x^* \in V^+$ cannot be verified, we follow the “no” arrow out of the box in the decision diagram. The lift to the original network means that the conclusions on the currently analyzed network also hold for the original network in an open parameter region.
### Monostationary networks

In all networks, the symbols $E$, $F$, $S$, $P$ refer to kinases, phosphatases, and substrates respectively. Taken with mass-action kinetics, all networks admit exactly one positive steady state in each stoichiometric compatibility class, which further is exponentially stable. Networks (1)-(4) model the phosphorylation of one substrate via different mechanisms. Network (5) models a substrate with two phosphorylation sites, while network (6) models the phosphorylation of two different substrates.

<table>
<thead>
<tr>
<th>Network</th>
<th>Reaction Rules</th>
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<tbody>
<tr>
<td>(1)</td>
<td>$S_0 + E \Rightarrow S_0E \rightarrow S_1 + E$ $S_1 + F \Rightarrow S_1F \rightarrow S_0 + F$</td>
</tr>
<tr>
<td>(2)</td>
<td>$S_0 + E \Rightarrow S_0E \rightarrow S_1 + E$ $S_1 + E \Rightarrow S_1E \rightarrow S_0 + E$</td>
</tr>
<tr>
<td>(3)</td>
<td>$S_0 + E_1 \Rightarrow S_0E_1 \rightarrow S_1 + E_1$ $S_0 + E_2 \Rightarrow S_0E_2 \rightarrow S_1 + E_2$ $S_1 + F \Rightarrow S_1F \rightarrow S_0 + F$</td>
</tr>
<tr>
<td>(4)</td>
<td>$S_0 + E_1 \Rightarrow S_0E_1 \rightarrow S_1 + E_1$ $S_0 + E_2 \Rightarrow S_0E_2 \rightarrow S_1 + E_2$ $S_1 + F_1 \Rightarrow S_1F_1 \rightarrow S_0 + F_1$ $S_1 + F_2 \Rightarrow S_1F_2 \rightarrow S_0 + F_2$</td>
</tr>
<tr>
<td>(5)</td>
<td>$S_0 + E_1 \Rightarrow S_0E_1 \rightarrow S_1 + E_1$ $S_1 + E_2 \Rightarrow S_1E_2 \rightarrow S_2 + E_2$ $S_1 + F_1 \Rightarrow S_1F_1 \rightarrow S_0 + F_1$ $S_2 + F_2 \Rightarrow S_2F_2 \rightarrow S_1 + F_2$</td>
</tr>
<tr>
<td>(6)</td>
<td>$S_0 + E \Rightarrow S_0E \rightarrow S_1 + E$ $P_0 + E \Rightarrow P_0E \rightarrow P_1 + E$ $S_1 + F_1 \Rightarrow S_1F_1 \rightarrow S_0 + F_1$ $P_1 + F_2 \Rightarrow P_1F_2 \rightarrow P_0 + F_2$</td>
</tr>
</tbody>
</table>

**Figure 3.** Monostationary networks. In all networks, the symbols $E$, $F$, $S$, $P$ refer to kinases, phosphatases, and substrates respectively. Taken with mass-action kinetics, all networks admit exactly one positive steady state in each stoichiometric compatibility class, which further is exponentially stable. Networks (1)-(4) model the phosphorylation of one substrate via different mechanisms. Network (5) models a substrate with two phosphorylation sites, while network (6) models the phosphorylation of two different substrates.

already determined for arbitrary $x \in \mathbb{R}^n_{>0}$, that is, only when it is necessary to evaluate at a positive steady state to decide the sign of $H_i$.

If some of the steps fail, then we consider reduced networks by removing either reactions that do not change the stoichiometric subspace, or intermediates. If stability is determined for a reduced network, then we conclude that the original network has at least the same number of positive steady states and stability properties as the reduced network in an open parameter region. In particular, if the reduced network has bistability, then so does the original network. For an expansion on how the open parameter regions arise when lifting rate constants and the properties of steady states, we refer the reader to [34, 42].

If stability is not determined for the chosen reduced network, then we consider another one, until all possibilities have been explored. If a reduced network does not display multistationarity, then further reductions on this network lead to networks that neither are multistationary. Being a reduced network of a reduced network establishes a partial order in the set of all reduced networks obtained by removal of intermediates or reactions. Therefore, a suitable strategy is to start investigating the largest networks. If one such network is not multistationary, all reduced networks smaller than this one can be disregarded.

We now use this approach on the remaining networks in Fig. 1. We consider a two substrate enzyme catalysis mechanism, comprising an enzyme $E$ that binds two substrates, $S_1$ and $S_2$, and catalyzes the reversible conversion to $P$. Taken with mass-action kinetics this network has one positive steady state in each stoichiometric compatibility class for any choice of rate constants $\kappa$ [14]. All but the last of the four Hurwitz determinants are positive for $x \in \mathbb{R}^6_{>0}$. We find a positive parametrization $\phi$ by solving the steady state equations in the concentrations of $ES_1$, $ES_2$, $ES_1S_2$ and $P$ using the procedure in [33], see Appendix B. After evaluating at $\phi$, $H_4$ becomes a rational function with only positive coefficients. Hence, all Hurwitz determinants are positive at a positive steady state, showing that the only positive steady state in each stoichiometric compatibility class is exponentially stable.

Next, we consider the gene transcription network in Fig. 1 with mass-action kinetics. For any choice of rate constants there exist at least two positive steady states in some stoichiometric compatibility class [14]. The computation of the Hurwitz determinants for arbitrary $x \in \mathbb{R}^7_{>0}$ gives that only $H_1$, $H_2$ are positive, but after evaluating at a positive parametrization, all but the last Hurwitz determinants are positive. We proceed to verify the assumptions of Theorem 1, see Appendix B. We obtain that the maximum number of positive steady states in any stoichiometric compatibility class is three, and that, whenever the network has one positive steady
state, then it is exponentially stable, and if it has three positive steady states, then two of them are exponentially stable and one is unstable. Hence bistability arises in this network.

**Bistability in cell signaling.** After having illustrated our approach with selected examples, we now investigate relevant cell signaling motifs. We follow the flow chart in Fig. 2 and perform all computations in Maple 2019 (see accompanying Maple file). To reduce computational cost, we compute first the Hurwitz determinants of a generic degree n polynomial (with unspecified coefficients), and then evaluate the determinant at the coefficients of $q_x$. We disregarded the Routh table from the package DynamicSystems as the computational cost was higher.

All networks in Fig. 4 are known to be monostationary under mass-action kinetics [32]. All Hurwitz determinants of $q_x$ are positive for positive $x$, without the need of a positive parametrization, see Appendix [33]. Hence for any choice of rate constants, each network in Fig. 4 has exactly one positive steady state in each stoichiometric compatibility class, which further is exponentially stable.

In the examples so far, we have not employed network reduction techniques, because all steps of the method could be carried through and the stability of a steady state depended only on the sign of the determinant of the Jacobian. This scenario is quite restrictive, as it implies that instabilities arise from a unique eigenvalue with positive real part.

The networks in Fig. 4 are all known to be multistationary with mass-action kinetics [32, 36]. Our method fails on the original networks: for network (c), the computation of the Hurwitz determinants was not possible in a regular PC due to lack of memory, and for the rest of the networks $H_{ij} > 0$ for $i \neq s$ does not hold. However, Theorem 1 applies to the reduced networks in Fig. 4. In particular, all reduced networks in Fig. 4 display bistability whenever they have three positive steady states. In this case, two of the steady states are exponentially stable and the third is unstable. Hence, after lifting stability to the original network using the results

<table>
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<tr>
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</tr>
<tr>
<td>$P_0 + S_1 \Leftrightarrow P_0S_1 \rightarrow P_1 + S_1$</td>
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**Figure 4.** Multistationary networks and reductions to assert bistability. (a) $E_1$ and $E_2$ are two conformations of a kinase catalyzing the phosphorylation of $S_0$ [35]. The reduced network is obtained by removing the intermediate $E_2S_0$ and all reverse reactions. (b) Cascade of two one-site modification cycles with the same phosphatase $F$. The reduced network is obtained by removing the intermediates $S_0E$ and $S_1F$ and all reverse reactions. (c) Cascade of one-site modification cycles where the same kinase $E$ acts in both layers. The reduced network is obtained by removing the reverse reactions and intermediates $S_0E_1$, $P_0S_1$ and $P_1F_2$. (d) Distribute and sequential phosphorylation of a substrate. The reduced network is obtained by removing the intermediates $S_1E$, $S_2F$ and $S_1F$ and all reverse reactions. (e) Phosphorylation of two substrates by the same kinase and phosphatase. The reduced network is obtained by removing the intermediates $S_0E$ and $P_1F$ and all reverse reactions. (f) Double phosphorylation of a substrate by the same kinase and two different phosphatases. The reduced network is obtained by removing all reverse reactions and the intermediates $S_1F_1$ and $S_2F_2$. Loans, and E Feliu


stated in Subsection 2.2 for all networks in Fig. 4, there is an open parameter region where the network has two exponentially stable positive steady states. We report only on a maximal reduced network that allows us to assert bistability for the original network. This means that, in practice, we might have checked several other reduced networks where Theorem 4 did not apply.

See Appendix B and the accompanying Maple file for details.

4. Proofs of the main results

We prove here the main results of our work. For completeness, we include the notation and the statements here, sometimes in an expanded form.

4.1. Proof of Proposition 1. Consider a matrix $R_0 \in \mathbb{R}^{n \times s}$ whose columns form a basis of $S$. This basis gives the system of coordinates in $S$. Therefore, given coordinates $z = (z_1, \ldots, z_n)$ in $S$, the vector $R_0z$ is the vector of coordinates in the canonical basis of $\mathbb{R}^n$. Conversely, selecting a matrix $R_1 \in \mathbb{R}^{s \times n}$ such that $R_1R_0 = I_{s \times s}$, we can write a vector $x \in S$ given in the canonical basis of $\mathbb{R}^n$, as a vector in local coordinates, by performing the product $R_1x$.

Using these matrices, the ODE system restricted to $(x^* + S) \cap \mathbb{R}^n_{\geq 0}$ in local $S$ coordinates is

$$\dot{x} = R_1f(R_0z + x^*)$$

after translating the steady state $x^*$ to the origin. The Jacobian matrix associated with this system at 0 is $R_1J_f(x^*)R_0$. The following proposition shows some basic properties of $R_1J_f(x^*)R_0$, and includes the results stated in Proposition 1. These properties follow from basic linear algebra and, for example, statements (i) and (ii) in Proposition 3 appear in [4, Appendix A]. We include a proof for completeness.

Proposition 3. Consider a reaction network with rate function $f(x) = Nv(x)$. Let $R_0 \in \mathbb{R}^{n \times s}$ and $R_1 \in \mathbb{R}^{s \times n}$ be matrices such that the columns of $R_0$ form a basis of the stoichiometric subspace $S$, and $R_1R_0 = I_{s \times s}$. The following statements hold:

(i) $R_1J_f(x^*)R_0 = LJ_v(x^*)R_0$, where $L \in \mathbb{R}^{s \times m}$ is the matrix such that $N = R_0L$. In particular, $R_1J_f(x^*)R_0$ does not depend on the choice of $R_1$.

(ii) If $R_0, R_0' \in \mathbb{R}^{n \times s}$ are two matrices with column span $S$, and $L, L'$ are as in (i) for $R_0, R_0'$ respectively, then the matrices $L'J_v(x^*)R_0'$ and $LJ_v(x^*)R_0$ are similar.

(iii) For a positive steady state $x^*$, the characteristic polynomials $p_{J_f}(\lambda)$ and $p_{LJ_v(x^*)R_0}(\lambda)$ satisfy $p_{J_f}(\lambda) = \lambda^{n-s}p_{LJ_v(x^*)R_0}(\lambda)$ for any choice of $R_0$.

(iv) The independent term of $p_{LJ_v(x^*)R_0}(\lambda)$ (or the coefficient of degree $n - s$ of $p_{J_f}(\lambda)$) equals $(-1)^s \det(J_{F_{\gamma}}(x^*))$, with $F_{\gamma}$ as in Eq. 3 for any choice of row-reduced matrix of conservation laws $W$.

Proof. (i) Since the columns of $N$ belong to $S$, we can uniquely write $N = R_0L$ with $L \in \mathbb{R}^{s \times m}$. Given that $J_f(x) = NJ_v(x)$, we have

$$R_1J_f(x^*)R_0 = R_1NJ_v(x^*)R_0 = R_1R_0LJ_v(x^*)R_0 = LJ_v(x^*)R_0.$$

(ii) Let $M \in \mathbb{R}^{s \times s}$ be the matrix of change of basis from $R_0$ to $R_0'$ such that $R_0M = R_0'$. From $N = R_0L' = R_0L$, it follows that $R_0ML' = R_0L$ and thus $L' = M^{-1}L$. This gives

$$L'J_v(x^*)R_0' = M^{-1}LJ_v(x^*)R_0M,$$

which implies that $L'J_v(x^*)R_0'$ and $LJ_v(x^*)R_0$ are similar.

(iii) Extend the matrix $R_0$ to a square matrix $R \in \mathbb{R}^{n \times n}$ by adding columns such that $R$ has full rank $n$. Then the eigenvalues of the matrices $Q = R^{-1}J_f(x^*)R$ and $J_f(x^*)$ coincide. We choose $R_1$ as the first $s$ rows of $R^{-1} = \begin{pmatrix} R_1 \\ R_1' \end{pmatrix}$. Then $R_1R_0 = I_{s \times s}$ and $R_1' = 0$. Since $\text{Im}(J_f(x^*)) \subseteq S$, the column span of $J_f(x^*)$ is contained in the column span of $R_0$, and thus $R_1'J_f(x^*) = 0$. Then, the matrix $Q$ has the form

$$Q = \begin{pmatrix} R_1 \\ R_1' \end{pmatrix} J_f(x^*) (R_0 \\ R_0') = \begin{pmatrix} R_1J_f(x^*) \\ 0 \end{pmatrix} (R_0 \\ 0) = \begin{pmatrix} R_1J_f(x^*)R_0 \\ 0 \end{pmatrix} = \begin{pmatrix} R_1J_f(x^*)R_0 \\ 0 \end{pmatrix}.$$
Clearly, the characteristic polynomial $p_Q(\lambda)$ is equal to
\[ \lambda^{n-s}P_R(J_f(x^*)R_0)(\lambda). \]

Using $R_1J_f(x^*)R_0 = LJ_1(x^*)R_0$, this concludes the proof of (iii).

(iv) The statement was proven in [57], Proposition 5.3. □

4.2. Proof of Proposition 2. We now turn into the proof of Proposition 2. In order to prove the identity in the statement, we make first three observations that rely on the definition of $\varphi$ and on the rule for multivariate functions:

1. By hypothesis, $\varphi(z) = (\varphi_1(z), \ldots, \varphi_n(z))$. Therefore, $\varphi'(z) = (\varphi'_1(z), \ldots, \varphi'_n(z))$.
2. Since $F_{T,\ell}(\varphi(z)) = 0$ for all $\ell \neq j$, we have that $(F_T \circ \varphi)(z)$ is a vector with zeros in every entry except for the $j$-th entry, which is equal to $(F_{T,j} \circ \varphi)(z)$. This implies that $(F_T \circ \varphi)^{\prime}(z)$ is also a vector with zero in every entry except in the $j$-th, that is equal to $(F_{T,j} \circ \varphi)^{\prime}(z)$.
3. By the chain rule $(F_T \circ \varphi)^{\prime}(z) = J_{F_T}(\varphi(z))\varphi^{\prime}(z)$.

From observations (2) and (3) we have
\[ J_{F_T}(\varphi(z))\varphi^{\prime}(z) = (0, \ldots, 0, (F_{T,j} \circ \varphi)^{\prime}(z), 0, \ldots, 0)^{tr}, \]
which means that the linear combination of the columns of $J_{F_T}(\varphi(z))$ given by the entries of $\varphi^{\prime}(z)$ is equal to the vector on the right side of the equation. Now, using observation (1), we compute $\det(J_{F_T}(x^*))$. Indeed, denoting by $J^k_{F_T}$ the $k$-th column of $J_{F_T}$, Eq. (12) gives
\[ \varphi'_i(z)J^k_{F_T}(\varphi(z)) = (0, \ldots, 0, (F_{T,j} \circ \varphi)^{\prime}(z), 0, \ldots, 0)^{tr} - \sum_{k=1, k \neq i}^{n} \varphi'_k(z)J^k_{F_T}(\varphi(z)). \]

Let $\tilde{J}_{F_T}(x^*)$ be the matrix obtained by replacing the $i$-th column of $J_{F_T}(\varphi(z))$ by the vector
\[ \left(0, \ldots, 0, \frac{(F_{T,j} \circ \varphi)^{\prime}(z)}{\varphi'_i(z)}, 0, \ldots, 0\right)^{tr}. \]

Then, $\det(J_{F_T}(\varphi(z)))$ and $\det(\tilde{J}_{F_T}(\varphi(z)))$ agree. Now, expanding the determinant of $\tilde{J}_{F_T}(\varphi(z))$ along the $i$-th column gives
\[ \det(J_{F_T}(x^*)) = \frac{(-1)^{i+j}}{\varphi'_i(z)}(F_{T,j} \circ \varphi)^{\prime}(z)\det(J_{F_T}(x^*), i, j). \]

This concludes the proof. □

4.3. Proof of Theorem 1 and a lemma. We start by proving Theorem 1. By the second hypothesis, the first $s - 1$ Hurwitz determinants are positive, so the stability only depends on the sign of the last Hurwitz determinant, which as discussed before, agrees with the sign of $(-1)^s \det(J_{F_T}(\varphi(z)))$. According to Proposition 2 it further coincides with the sign of $\frac{(-1)^{s+i+j}}{\varphi'_i(z)}(F_{T,j} \circ \varphi)^{\prime}(z)\det(J_{F_T}(\varphi(z)), i, j)$. Since $\det(J_{F_T}(\varphi(z)), i, j)$ has a constant sign for every $z \in \mathcal{E}$, the sign of the last Hurwitz determinant changes when the sign of $(F_{T,j} \circ \varphi)^{\prime}(z)$ does, and this is the derivative of a univariate differentiable function whose real positive roots $z_1 < \ldots < z_k$ have multiplicity one and are ordered in an increasing way. Given that $(F_{T,j} \circ \varphi)$ is differentiable, the sign of its derivative evaluated at consecutive roots alternates, that is $(F_{T,j} \circ \varphi)(z_1)' > 0, (F_{T,j} \circ \varphi)(z_2)' > 0, \ldots$ and $(F_{T,j} \circ \varphi)(z_2)' < 0, (F_{T,j} \circ \varphi)(z_4)' < 0, \ldots$ or the other way around.

In our setting this means that, once the sign of $(F_{T,j} \circ \varphi)'(z_k)$ is multiplied by $(-1)^{s+i+j}$ and by the sign of $\frac{1}{\varphi'_i(z)}\det(J_{F_T}(\varphi(z)), i, j)$, either $\varphi(z_1), \varphi(z_3), \ldots$ are exponentially stable and $\varphi(z_2), \varphi(z_4), \ldots$ are unstable, or the other way around. In particular, if the sign of $(F_{T,j} \circ \varphi)(z_k)'$ times the sign of $(-1)^{s+i+j}\frac{1}{\varphi'_i(z)}\det(J_{F_T}(\varphi(z)), i, j)$ is positive, then $\varphi(z_1)$ is exponentially stable. □
We include a simple lemma to ensure that, in the setting of Theorem \[\text{[4]}\] only the numerator of \(F_{T,j} \circ \varphi\) needs to be considered.

**Lemma 1.** Under the assumptions of Theorem \[\text{[4]}\], assume \((F_{T,j} \circ \varphi)(z) = \frac{a(z)}{b(z)}\) is a rational function in \(z\) such that \(b(z)\) is positive in \(\mathcal{E}\). Then the zeros of \((F_{T,j} \circ \varphi)(z)\) agree with the roots of \(a(z)\) and the sign of \((F_{T,j} \circ \varphi)'(z')\), and \(a'(z')\) agree for all \(z' \in \mathcal{E}\) such that \(a(z') = 0\).

**Proof.** The first part is straightforward, since the denominator of \((F_{T,j} \circ \varphi)(z)\) does not vanish in \(\mathcal{E}\). For the second part, we have \(F_{T,j}(\varphi(z')) = \frac{a'(z'\bar{z})b(z') - a(z')b'(z')}{a(z')^2} = \frac{a'(z')}{\bar{a}(\bar{z})},\) and the conclusion follows from the fact that \(b(z') > 0\).

## 5. Computational challenges

In our context, the Hurwitz determinants are symbolic and depend on \(\kappa\) and \(x\) or \(\xi\). Their computation requires the storage of functions with many terms, which easily becomes unfeasible in a regular PC. For example, for network (6) in Fig. 3, \(H_1\) and \(H_5\) are polynomials in \(\kappa\) and \(x\) with respectively 1,732,192 and 37,609,352 monomials, before the evaluation at a parametrization.

For medium sized networks, some tricks can be applied under mass-action. A first strategy is to parametrize the positive steady state variety using convex parameters introduced by Clarke \[12\], \[27\]. This conversion may reduce the number of parameters, mainly if the network has few reversible reactions.

The second strategy corresponds to encode a monomial \(\eta x_1^{a_1} \cdots x_n^{a_n}\) as an \((n+1)\)-tuple \((\eta, a_1, \ldots, a_n)\), where \(\eta\) is a rational function in \(\kappa\). We exploit relations among the \(H_i\) obtained by expanding recursively along the last column of the submatrix of \(H\) giving rise to \(H_i\). For example, we have a relation

\[
H_3 = a_{s-3}H_2 - a_{s-1}(a_{s-4}H_1 + a_s a_{s-5})
\]

that holds for a generic polynomial. Assume \(H_i\) is written as a sum of terms \(H_{i,1}, \ldots, H_{i,t}\) that can be computed. Normally these terms arise from the coefficients of the characteristic polynomial and Hurwitz determinants \(H_j\) with \(j < i\), like the terms \(a_{s-3}H_2\) and \(a_{s-1}(a_{s-4}H_1 + a_s a_{s-5})\) for \(H_3\). We identify the monomials of each term with their corresponding \((n+1)\)-tuple and gather them into a list \(L_1\). Then, we create a list \(L_2\) of the tuples of \(L_1\) for which \(\eta\) might not be a positive function of \(\kappa\). Note that there might be repeated monomials in \(H_{i,1}, \ldots, H_{i,t}\), whose coefficients we need to add. To this end, for each element \((\eta^*, \alpha_1^*, \ldots, \alpha_n^*)\) in \(L_2\), we define the set

\[
\tilde{L}(\eta^*, \alpha_1^*, \ldots, \alpha_n^*) := \{(\eta, \alpha_1, \ldots, \alpha_n) \in L_1 \mid \alpha_1 = \alpha_1^*, \ldots, \alpha_n = \alpha_n^*\}.
\]

Then

\[
\eta = \sum_{(\eta, \alpha_1, \ldots, \alpha_n) \in \tilde{L}(\eta^*, \alpha_1^*, \ldots, \alpha_n^*)} \eta
\]

is the coefficient of \(x_1^{\alpha_1} \cdots x_n^{\alpha_n}\) in \(H_i\), and we inspect its sign. With this process we consider only the coefficients that might generate negative values of \(H_i\) as they might be negative in some \(H_{i,j}\). If all coefficients \(\eta\) are nonnegative, then so is \(H_i\). This procedure requires substantially less memory, but it might take time as lists can be long. With this strategy we have determined the sign of \(H_5\) in networks (5) and (6) in Fig. 3.

The third strategy applies to a special situation, namely when there exists a monomial positive parametrization

\[
\phi(\xi) = \beta_i \xi_1^{b_{i1}} \cdots \xi_d^{b_{id}}, \quad i = 1, \ldots, n,
\]

with \(\beta\) depending on the rate constants \[51\]. Assume \(H_i\) can be computed for \(x \in \mathbb{R}_{>0}^n\), but evaluating at a parametrization and expanding the resulting polynomial to inspect its sign encounters memory issues. Let \(B = (b_{ij}) \in \mathbb{Z}^{d \times n}\) be the matrix arising from the exponents of the monomial parametrization, and write \((\xi^B) = \xi_1^{b_{11}} \cdots \xi_d^{b_{id}}\).

After evaluation at \(\phi(\xi)\), a monomial \(\eta x_1^{a_1} \cdots x_n^{a_n}\) of \(H_i\) becomes

\[
\eta (\beta_1 \xi_1^{a_{11}} \cdots \xi_d^{a_{1d}})^{\alpha_1} \cdots (\beta_n \xi_1^{a_{n1}} \cdots \xi_d^{a_{nd}})^{\alpha_n} = \eta \beta_1^{a_1} \cdots \beta_n^{a_n} \xi^{Ba}.
\]
Hence, to compute \( H_i(\phi(\xi)) \) we first write each monomial of \( H_i(x) \) as an \((n + d + 1)\)-tuple given by \((\eta, \alpha_1, \ldots, \alpha_n, 0, \ldots, 0)\). Then, we record the evaluation of each monomial with the \((n + d + 1)\)-tuple

\[
(\eta, \alpha_1, \ldots, \alpha_n, (B\alpha)_1, \ldots, (B\alpha)_d).
\]

This generates a list \( L_1 \) of \((n + d + 1)\)-tuples. We define \( L_2 \) as the set of tuples of \( L_1 \) for which \( \eta \) might not be a positive function of \( \kappa \). Then, similar to the procedure explained above, for each \( t = (\eta^*, \alpha_1^*, \ldots, \alpha_n^*, (B\alpha)_1^*, \ldots, (B\alpha)_d^*) \in L_2 \), we consider all tuples that give rise to the same monomial in \( \xi \), namely, we define

\[
L_t := \{(\eta, \alpha_1, \ldots, \alpha_n, (B\alpha)_1, \ldots, (B\alpha)_d) \in L_1 \mid B\alpha = B\alpha^* \}.
\]

We compute now the coefficient of \( \xi^{B\alpha} \), which is

\[
\eta = \sum_{(\eta, \alpha_1, \ldots, \alpha_n, (B\alpha)_1, \ldots, (B\alpha)_d) \in L_t} \eta^{\alpha_1} \cdots \alpha_n.
\]

We determine its sign to decide whether \( H_i \) is positive.

With this approach, we computed the Hurwitz determinants of networks (a), (b), (d) and (e) in Fig. 4. However, \( H_{s-1} \) is not positive and Theorem 1 does not apply.

To verify that a polynomial with both positive and negative coefficients attains both signs, one can study the associated Newton polytope, as employed in the context of reaction networks in [14, 17] to cite a few. To assert that a polynomial only attains positive values despite having negative coefficients, one can employ techniques from sum-of-squares [9] and polynomial optimization via sums of nonnegative circuit polynomials [25, 30, 41]. Another approach, building on similar ideas but with a specific focus on reaction networks, was introduced in [49]. However, the size of the polynomials we encounter makes these approaches challenging.

**Discussion**

All the steps of our approach to determine the stability are symbolic, and therefore provide computer-assisted proofs for bistability. In the most favorable scenario where Theorem 1 applies, the number of unstable and exponentially stable steady states is completely determined, and question (3) in the Introduction is answered. In particular, if the reduced univariate equation has at least three solutions and the first steady state is exponentially stable, the parameter region of bistability agrees with the parameter region giving three positive steady states. Finding the latter poses a simpler (though still hard) challenge, which can be (partially) addressed using recent methods [8, 14].

Under mass-action kinetics, reduction of the steady state equations to one polynomial can in principle be achieved using Gröbner bases and invoking the Shape Lemma [18]. However, positivity is not addressed, and the interval \( \xi^* \) in Proposition 2 is rarely explicit. Reduction to one polynomial arises often after exploiting the inherent linearity the equations have [33].

In our procedure, the Hurwitz criterion can be replaced by other criteria of algebraic nature, namely the Liénard-Chipart criterion in [21] or checking whether the matrix \( Q_{x^*} \) is both a P-matrix and sign-symmetric. However, these criteria can only be used to assert exponential stability (see Appendix A, where these criteria are applied to the network in Eq. (1)).

We have illustrated with numerous realistic examples that our approach determines bistability after performing network reduction. To our knowledge, this is a new result for all networks in Fig. 4 but network (d). For this one, bistability was formally proven in [39] using methods from geometric singular perturbation theory and the accurate study of a reduced network. We see our approach as a big step towards the automatic detection of bistability in open parameter regions, which relies on purely algebraic manipulations instead of advanced analytic arguments. Although the approach is applicable to arbitrary ODE systems, the special structure of the systems arising from reaction networks, specifically linearity, the existence of conservation laws and reduction techniques, make the approach particularly suited for this scenario.

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SYMBOLIC PROOF OF BISTABILITY IN REACTION NETWORKS

REFERENCES


In this appendix we discuss two additional criteria to decide the stability of the steady states.

**Definition 1.**

- The Bezout matrix of two polynomials \( h(x) = h_n x^n + h_{n-1} x^{n-1} + \cdots + h_1 x + h_0 \) and \( g(x) = g_m x^m + g_{m-1} x^{m-1} + \cdots + g_1 x + g_0 \) with \( n \geq m \), denoted by \( B_{h,g} \), is defined as the representation matrix of the bilinear form

\[
B(h, g; x, y) = \frac{h(x)g(y) - h(y)g(x)}{x - y} = \sum_{i,j=0}^{n-1} b_{ij} x^i y^j,
\]

that is, \( B_{h,g} := (b_{ij}) \). This is a symmetric matrix of size \( n \times n \).

- A square matrix \( A \in \mathbb{R}^{n \times n} \) is called a P-matrix if all its principal minors are positive, that is, \( \det(A_{I,I}) > 0 \) for every subset \( I \subseteq \{1, \ldots, n\} \). If \( A \) is symmetric, this is equivalent to \( A \) being positive definite.

We now present the first additional stability criterion.

**Criterion 2** (Liénard-Chipart). *All the roots of a polynomial \( p(x) = x^s + p_{s-1} x^{s-1} + \cdots + p_1 x + p_0 \) with \( p_i \in \mathbb{R} \) and \( p_0 \neq 0 \) have negative real part if and only if, after writing \( p(x) = h(x^2) + x g(x^2) \), the Bezout matrix \( B_{h,g} \) of \( h \) and \( g \) is positive definite and \( p_i > 0 \) for \( i = 1, \ldots, s \).

In this criterion the polynomials \( h \) and \( g \) are associated with the even and odd parts of \( p \) respectively. Note that the degrees of \( h \) and \( g \) are at most \( \lfloor \frac{s}{2} \rfloor \), therefore the size of \( B_{h,g} \) is \( \lfloor \frac{s}{2} \rfloor \). Additionally, since \( B_{h,g} \) is symmetric, \( B_{h,g} \) is positive definite if and only if it is a P-matrix.

Unlike the Hurwitz criterion, Liénard-Chipart does not give a result regarding instability. If \( B_{h,g} \) is not a P-matrix, it is not possible to determine whether the eigenvalues that do not have negative real part, have positive or zero real part.

In order to apply the criterion to Example 1 we write \( q_x(\lambda) \) as \( h(\lambda^2) + \lambda g(\lambda^2) \), with

\[
h(\lambda) = \lambda + \kappa_1 \kappa_2 x_2 + \kappa_2 \kappa_3 x_3 + \kappa_1 \kappa_3 \quad \text{and} \quad g(\lambda) = \kappa_2 x_2 + \kappa_3 x_3 + \kappa_1 + \kappa_3.
\]

We now expand on other stability criteria that could be used in our procedure instead of the Hurwitz criterion, and in Appendix B we provide details of the analysis of the networks in Figures 3 and 4.

**APPENDICES**

**APPENDIX A. OTHER ALGEBRAIC CRITERIA FOR STABILITY**

In this appendix we discuss two additional criteria to decide the stability of the steady states. Similarly to the Hurwitz criterion, the Liénard-Chipart criterion in [21], determines whether all the roots of a polynomial have negative real part, and requires a smaller amount of computations than the Hurwitz criterion. Before introducing the criterion, we need some ingredients.

**Definition 1.**

- The Bezout matrix of two polynomials \( h(x) = h_n x^n + h_{n-1} x^{n-1} + \cdots + h_1 x + h_0 \) and \( g(x) = g_m x^m + g_{m-1} x^{m-1} + \cdots + g_1 x + g_0 \) with \( n \geq m \), denoted by \( B_{h,g} \), is defined as the representation matrix of the bilinear form

\[
B(h, g; x, y) = \frac{h(x)g(y) - h(y)g(x)}{x - y} = \sum_{i,j=0}^{n-1} b_{ij} x^i y^j,
\]

that is, \( B_{h,g} := (b_{ij}) \). This is a symmetric matrix of size \( n \times n \).

- A square matrix \( A \in \mathbb{R}^{n \times n} \) is called a P-matrix if all its principal minors are positive, that is, \( \det(A_{I,I}) > 0 \) for every subset \( I \subseteq \{1, \ldots, n\} \). If \( A \) is symmetric, this is equivalent to \( A \) being positive definite.

We now present the first additional stability criterion.

**Criterion 2** (Liénard-Chipart). *All the roots of a polynomial \( p(x) = x^s + p_{s-1} x^{s-1} + \cdots + p_1 x + p_0 \) with \( p_i \in \mathbb{R} \) and \( p_0 \neq 0 \) have negative real part if and only if, after writing \( p(x) = h(x^2) + x g(x^2) \), the Bezout matrix \( B_{h,g} \) of \( h \) and \( g \) is positive definite and \( p_i > 0 \) for \( i = 1, \ldots, s \).

In this criterion the polynomials \( h \) and \( g \) are associated with the even and odd parts of \( p \) respectively. Note that the degrees of \( h \) and \( g \) are at most \( \lfloor \frac{s}{2} \rfloor \), therefore the size of \( B_{h,g} \) is \( \lfloor \frac{s}{2} \rfloor \). Additionally, since \( B_{h,g} \) is symmetric, \( B_{h,g} \) is positive definite if and only if it is a P-matrix.

Unlike the Hurwitz criterion, Liénard-Chipart does not give a result regarding instability. If \( B_{h,g} \) is not a P-matrix, it is not possible to determine whether the eigenvalues that do not have negative real part, have positive or zero real part.

In order to apply the criterion to Example 1 we write \( g_x(\lambda) \) as \( h(\lambda^2) + \lambda g(\lambda^2) \), with

\[
h(\lambda) = \lambda + \kappa_1 \kappa_2 x_2 + \kappa_2 \kappa_3 x_3 + \kappa_1 \kappa_3 \quad \text{and} \quad g(\lambda) = \kappa_2 x_2 + \kappa_3 x_3 + \kappa_1 + \kappa_3.
\]
The Bezout matrix is then $B_{h,g} = (x_2 + x_3)\kappa_2 + \kappa_1 + \kappa_3$, which is clearly a P-matrix. By the Liénard-Chipart criterion, we conclude that all the roots of $q_x$ have negative real part, recovering thereby that the only positive steady state in each stoichiometric compatibility class is exponentially stable.

The Liénard-Chipart criterion is the most efficient if we consider the amount of determinants that have to be computed to reach a decision. Given that the Bezout matrix is symmetric, to check that is positive definite, it is only necessary to compute the principal minors and check whether they are positive. Thus the required amount of determinants is at most $\sum_{i=0}^{\left\lceil \frac{n+1}{2} \right\rceil} (\left\lceil \frac{n+1}{2} \right\rceil^i)$. Although this criteria computes the smallest amount of determinants, for some examples, the entries of the Bezout matrix are larger than the entries of the Jacobian. In those cases the memory of a regular PC is still not enough to store the computations.

We conclude the list of stability criteria of algebraic nature with one more criterion, which does not rely on the computation of the characteristic polynomial. For a square matrix $A$, we say that $A$ is sign symmetric if $\det(A_{I,J}) \det(A_{J,I}) \geq 0$ for every $I,J \subset \{1, \ldots, n\}$ with the same cardinality.

**Criterion 3 (P-matrices that are sign symmetric).** If a square matrix $A$ is both a P-matrix and sign symmetric, then all its eigenvalues have positive real part.

With this criterion, proved in [11], if $-A$ is a P-matrix and sign-symmetric, then all its eigenvalues have negative real part. For reaction networks, we apply the criterion to the matrix $-Q_x$. In Example 1 we compute the minors of size 1 and 2 of $A = -Q_x$. The minors of size 1 are the entries of the matrix, which are all positive. The only minor of size 2 is

$$\det(A) = \kappa_1 \kappa_2 x_2 + \kappa_2 \kappa_3 x_3 + \kappa_1 \kappa_3.$$ 

All minors are polynomials that are positive for all $x \in \mathbb{R}^4_{>0}$ and positive $\kappa$. Therefore $-Q_x$ is a P-matrix and sign symmetric. Hence, with this new criterion we recover the conclusion that the only positive steady state is exponentially stable.

While the Hurwitz and Liénard-Chipart criteria are applied to the characteristic polynomial of $LJ_v(x^*)R_0$, which is independent of the choice of $R_0$, Criterion 3 is applied directly to the matrix $-LJ_v(x^*)R_0$. By Proposition 1, two different choices of $R_0$ give rise to two similar matrices. However, the properties of being P-matrix and sign symmetric are not preserved on similar matrices. As a small example consider

$$A = \begin{pmatrix} 2 & 1 \\ 3 & 4 \end{pmatrix} \quad \text{and} \quad B = \begin{pmatrix} -1 & -4 \\ 3 & 7 \end{pmatrix};$$

these matrices are similar through $M = \begin{pmatrix} 1 & 1 \\ 0 & 1 \end{pmatrix}$, but $A$ is both a P-matrix and sign symmetric and $B$ is neither.

A comparison of the amount of operations required for each stability criterion shows that deciding whether a matrix is a P-matrix and sign-symmetric requires the largest amount of operations. In this case, all $\sum_{i=1}^{s} \binom{s}{i}^2$ minors of the matrix must be computed, which requires the storage of a large amount of information given that the entries of the Jacobian are typically polynomials.

**Appendix B. Examples**

In this section we provide extra details on the study of the networks in Figures 3 and 4. For more details on the computations, we refer the reader to the accompanying Maple file. Concentrations are denoted by corresponding lower case letters: the concentration of species $X_i$ is denoted by $x_i$. 
B.1. Two substrate enzyme catalysis (Fig. 1). We consider the following network with mass-action kinetics

\[
E + S_1 \xrightarrow{k_1} E_S_1 \quad E + S_2 \xrightarrow{k_2} E_S_2 \quad S_2 + ES_1 \xrightarrow{s_{12}} ES_2 S_2
\]

This network consists of an enzyme \(E\) that binds two substrates, \(S_1\) and \(S_2\), in order to catalyze the reversible conversion to the product \(P\). The binding is unordered. It was proven in [14] that this network has a unique steady state in each stoichiometric compatibility class for each set of reaction rate constants. We now prove that this steady state is exponentially stable. First, denote the species as \(X_1 = E, X_2 = S_1, X_3 = ES_1, X_4 = S_2, X_5 = ES_2, X_6 = ES_1 S_2\) and \(X_7 = P\). The ODE system is

\[
\begin{align*}
\frac{dx_1}{dt} &= -k_1 x_1 x_2 - k_3 x_3 x_4 - k_4 x_3 x_5 + k_5 x_6 + k_7 x_6 \\
\frac{dx_2}{dt} &= -k_1 x_1 x_2 - k_4 x_3 x_5 + k_2 x_3 + k_7 x_6 \\
\frac{dx_3}{dt} &= k_1 x_1 x_2 - k_3 x_3 x_4 - k_2 x_3 + k_6 x_6 \\
\frac{dx_4}{dt} &= -k_3 x_3 x_4 + k_5 x_6 + k_7 x_6 \\
\frac{dx_5}{dt} &= -k_3 x_3 x_4 + k_5 x_3 x_4 + k_4 x_5 + k_6 x_6 \\
\frac{dx_6}{dt} &= -k_3 x_3 x_4 + k_5 x_3 x_4 + k_4 x_5 + k_6 x_6 \\
\frac{dx_7}{dt} &= -k_1 x_1 x_2 + k_4 x_3 x_5 - k_3 x_3 x_4 - k_2 x_3 + k_6 x_6 \\
\end{align*}
\]

and the conservation laws are

\[x_1 + x_3 + x_5 + x_6 = T_1, \quad x_2 + x_3 + x_6 + x_7 = T_2 \quad \text{and} \quad x_4 + x_5 + x_6 + x_7 = T_3.\]

Here \(s = 4\). We compute \(q_x\) and the Hurwitz determinants in Maple, and find that all but the last have coefficients positive, and thus are positive. We find next a positive parametrization by solving the steady state equations in the variables \(x_3, x_5, x_6, x_7\) following the methods proposed in [14, 33]:

\[
\begin{align*}
x_3 &= \frac{x_1 x_2 (k_k k_3 (k_1 x_2 + k_3 x_4) + k_4 k_4 (k_6 + k_7))}{k_2 k_6 k_8 x_3 + k_4 k_5 k_7 x_4 + k_2 k_6 k_8 + k_2 k_4 k_7} \\
x_5 &= \frac{x_1 x_4 (k_2 k_7 (k_1 x_2 + k_3 x_4) + k_2 k_3 (k_6 + k_7))}{k_2 k_6 k_8 x_2 + k_4 k_5 k_7 x_4 + k_2 k_6 k_8 + k_2 k_4 k_7} \\
x_6 &= \frac{x_1 x_4 (k_2 k_7 (k_1 x_2 + k_3 x_4) + k_2 k_3 (k_6 + k_7))}{k_2 k_6 k_8 x_2 + k_4 k_5 k_7 x_4 + k_2 k_6 k_8 + k_2 k_4 k_7} \\
x_7 &= \frac{x_1 x_4 (k_2 k_7 (k_1 x_2 + k_3 x_4) + k_2 k_3 (k_6 + k_7))}{k_2 k_6 k_8 x_2 + k_4 k_5 k_7 x_4 + k_2 k_6 k_8 + k_2 k_4 k_7} \\
\end{align*}
\]

After evaluation of the independent term of \(q_x\) at the parametrization, \(H_4\) becomes positive. We conclude that for any choice of reaction rate constants, the network for two substrate enzyme catalysis has exactly one positive steady state in each stoichiometric compatibility class, which is exponentially stable.

B.2. Gene transcription network (Fig. 1). We consider the following network:

\[
\begin{align*}
X_1 &\xrightarrow{k_1} X_1 + P_1 \\
X_2 &\xrightarrow{k_2} X_2 + P_2 \\
P_1 &\xrightarrow{k_3} 0 \\
P_2 &\xrightarrow{k_4} 0 \\
X_2 + P_1 &\xrightarrow{k_5} X_2 P_1 \\
2P_2 &\xrightarrow{k_7} P_2 P_2 \\
X_1 + P_2 P_2 &\xrightarrow{k_9} X_1 P_2 P_2.
\end{align*}
\]

We denote the species as \(X_1 = X_1, X_2 = X_2, X_3 = P_1, X_4 = P_2, X_5 = X_2 P_1, X_6 = P_2 P_2, \) and \(X_7 = X_1 P_2 P_2\). Additionally, we are under the assumption of mass-action kinetics. It was proven in [14] that for each set of positive reaction rate constants there is a stoichiometric compatibility class that contains at least two positive steady states. The ODE system is

\[
\begin{align*}
\frac{dx_1}{dt} &= -k_9 x_1 x_6 + k_7 x_7 \\
\frac{dx_2}{dt} &= -k_5 x_2 x_3 + k_1 x_1 - k_3 x_3 + k_6 x_5 \\
\frac{dx_3}{dt} &= k_5 x_2 x_3 - k_6 x_5 \\
\frac{dx_4}{dt} &= k_5 x_2 x_3 - k_6 x_5 \\
\frac{dx_5}{dt} &= k_5 x_2 x_3 - k_6 x_5 \\
\frac{dx_6}{dt} &= k_5 x_2 x_3 - k_6 x_5 \\
\frac{dx_7}{dt} &= k_5 x_2 x_3 - k_6 x_5 \\
\end{align*}
\]
and the conservation laws are \( x_1 + x_7 = T_1 \) and \( x_2 + x_5 = T_2 \). These give rise to the function \( F_T(x) \):

\[
F_T(x) = \begin{pmatrix}
  x_1 + x_7 - T_1 \\
  x_2 + x_5 - T_2 \\
  -k_5x_2x_3 + k_4x_1 - k_3x_3 + \kappa_6x_5 \\
  -2k_7x_2^2 + k_5x_2 - k_4x_4 + 2k_8x_6 \\
  k_5x_2x_3 - \kappa_6x_5 \\
  k_7x_4^2 - k_9x_1x_6 - \kappa_8x_6 + \kappa_{10}x_7 \\
  k_9x_1x_6 - \kappa_{10}x_7
\end{pmatrix}.
\]

Here \( s = 5 \). We find \( q_z \) and compute the 5 Hurwitz determinants for \( x \in \mathbb{R}^7_0 \). We find that \( H_3, H_4, H_5 \) have coefficients of both signs. We proceed to find a parametrization by solving the steady state equations in \( x_3, \ldots, x_7 \), which gives:

\[
\begin{align*}
  x_3 &= \frac{k_1x_1}{k_3}, & x_4 &= \frac{k_2x_2}{k_4}, & x_5 &= \frac{k_1k_5x_1x_2}{k_3k_6}, & x_6 &= \frac{k_2k_7x_2^2}{k_4^6k_8^5}, & x_7 &= \frac{k_2k_7k_9x_1x_2^2}{k_4^7k_8k_{10}}.
\end{align*}
\]

After evaluating \( H_3, H_4, H_5 \) in this parametrization, \( H_3 \) and \( H_4 \) become rational functions in \( x_1, x_2 \) and \( \kappa \) with all coefficients positive. Hence they are positive as well.

This means that the stability of the steady state is determined by the sign of \( H_5 \). We check whether we can apply Theorem 1. By solving \( F_T(x) = 0 \) in \( x_2, \ldots, x_7 \) after removal of the conservation law with total amount \( T_1 \), we obtain

\[
\begin{align*}
  x_2 &= \frac{k_3k_6T_2}{k_1k_5x_1 + k_3k_6}, & x_3 &= \frac{k_1x_1}{k_3}, & x_4 &= \frac{k_2k_5k_6T_2}{(k_1k_5x_1 + k_3k_6)k_4}, \\
  x_5 &= \frac{k_1k_5k_6T_2}{k_1k_5x_1 + k_3k_6}, & x_6 &= \frac{k_2k_5k_6^2k_7T_2^2}{(k_1k_5x_1 + k_3k_6)k_4^6k_8^5}, & x_7 &= \frac{k_2k_5k_6^2k_7k_9k_1x_2^2T_2^2}{(k_1k_5x_1 + k_3k_6)k_4^7k_8k_{10}}.
\end{align*}
\]

These expressions define \( \varphi \), with \( z = x_1, \varphi_1(z) = z \) and \( E = \mathbb{R}_{>0} \). By inserting these expressions into the conservation law with \( T_1 \), we conclude that the solutions of \( F_T(x) = 0 \) are in one to one correspondence with the zeroes of the function

\[
(F_{T,1} \circ \varphi)(z) = \frac{1}{\det(J_{F_T}(\varphi(z)),1,1)} \det(J_{F_T}(\varphi(z)),(2,\ldots,7),(2,\ldots,7)) = -(k_1k_5x_1 + k_3k_6)k_4k_6k_{10} < 0.
\]

By Theorem 1, the sign of \( (11) \) is positive since \( s + i + j = 7 \) and the sign of \( (F_{T,1} \circ \varphi)(z_1) \) is positive as the independent term of the numerator of \( (F_{T,1} \circ \varphi)(z_1) \) is negative. Therefore, the stability of the steady states alternate with \( z \) starting with an exponentially stable steady state. Specifically, if a stoichiometric compatibility class has one positive steady state, then it is exponentially stable. If it has three positive steady states, then two of them are exponentially stable and the other one is unstable. Bistability is guaranteed whenever the network has three positive steady states.

### B.3. Monostationary networks from Fig. 3

Networks (1) to (4) are straightforward to analyze, since all coefficients of the Hurwitz determinants in \( x \) and \( \kappa \) are positive; hence the Hurwitz determinants are positive for all \( x \in \mathbb{R}^6_0 \) and \( \kappa \in \mathbb{R}^5_{>0} \).

For networks (5) and (6) in Fig. 3, the computation was interrupted as it took long. In both networks \( s = 6 \), and the first four Hurwitz determinants could be computed. These determinants are polynomials in \( \kappa \) and \( x \) with positive coefficients, thus they are positive for every positive steady state. In order to compute \( H_5 \), we followed the second strategy explained in Section 5.

We rely on an identity that holds for the Hurwitz determinants of a generic polynomial of degree 6. Namely, for a generic polynomial \( h(t) = a_0t^6 + a_5t^5 + a_4t^4 + a_3t^3 + a_2t^2 + a_1t + a_0 \), the fifth Hurwitz determinant can be written in terms of the previous ones as follows

\[
H_5 = a_1H_4 + a_0(-a_0a_5^2 + a_1a_5H_2 - a_3H_3).
\]
With this identity, we analyze the sign of the coefficients of \( H_5 \) by studying separately the coefficients of \( A = a_0(-a_0a_3^3 + a_1a_5H_2 - a_3H_3) \) and \( B = a_1H_4 \) after substituting \( a_i \) for the coefficient of \( \lambda^i \) in \( q_x(\lambda) \). Note that the coefficients of \( B \) are positive because both \( a_1 \) and \( H_4 \) are polynomials with positive coefficients. We proceed to find the coefficients of \( H_5 \) by selecting the terms of \( A \) that might be negative. We actually found that all coefficients of \( A \) are polynomials in \( \kappa \) with some negative coefficients. When matched with monomials in \( B \), all negative coefficients cancel out, confirming that \( H_5 \) is positive for all positive \( \kappa \) and \( x \). Therefore, \( H_5 \) is positive for every positive steady state. Knowing this about \( H_5 \), we also conclude that \( H_6 = a_0H_5 \) is a polynomial with positive coefficients and the unique steady state in each stoichiometric compatibility class is exponentially stable for every set of parameters. Note that in these computations we do not need to evaluate at a positive parametrization, meaning all Hurwitz determinants are positive for arbitrary positive \( \kappa \) and \( x \).

In networks (5) and (6), this strategy to compute \( H_5 \) meant that we were analyzing only 24,196 and 27,982 coefficients instead of 37,319 and 36,970 coefficients respectively.

**B.4. Multistationary networks from Fig. 4** We consider now the networks in Fig. 4 which all are known to be multistationary. We sketch here why the procedure fails for each network, and how it applies to the reduced network. To certify multistationarity for the reduced networks, we will apply the method from [13], which consists of finding values for the rate constants and concentration variables such that \( \det(J_{\phi}(\phi)) = 0 \), where \( \phi \) is a parametrization of the steady states.

**Network (a).** This network is the combination of two one-site modification cycles where the same kinase \( E \) activates the phosphorylation process and two different phosphates \( F_1 \) and \( F_2 \) catalyze the dephosphorylation process:

\[
\begin{align*}
S_0 + E \xrightarrow{\kappa_1} S_0E & \xrightarrow{\kappa_3} S_1 + E \\
S_1 + F_1 \xrightarrow{\kappa_5} S_1F_1 & \xrightarrow{\kappa_9} S_0 + F_1
\end{align*}
\]

The species are renamed as \( S_0 = X_1, S_1 = X_2, S_2 = X_3, E = X_4, F_1 = X_5, F_2 = X_6, S_0E = X_7, S_1E = X_8, S_1F_1 = X_9, S_2F_2 = X_{10} \). Since the polynomial \( q_x \) has degree 6, we need to compute 6 Hurwitz determinants. These determinants were computed and their signs were analyzed up to \( H_{14} \), and they have positive coefficients. However, the analysis of the sign of \( H_5 \) was interrupted as it took long and it was not possible to store the polynomial in the expanded format in a regular PC. To compute and study this determinant more effectively, we use a monomial positive parametrization \( \phi \) of the steady state variety, which, in the notation given in Section 5, corresponds to

\[
\beta = \left( \frac{(k_2 + k_3)(k_5 + k_6)k_7k_9k_{10}k_{11}k_{12}}{k_1k_3k_4k_6(k_8 + k_9)(k_{11} + k_{12})}, \frac{(k_5 + k_6)k_{10}k_{12}}{k_3k_4k_6(k_8 + k_9)(k_{11} + k_{12})}, 1, 1, 1, 1, 1, \frac{(k_5 + k_6)k_7k_9k_{10}k_{12}}{k_3k_4k_6(k_8 + k_9)(k_{11} + k_{12})}, \frac{k_{10}}{k_{11} + k_{12}} \right),
\]

\[
B = \begin{bmatrix}
1 & 1 & 1 & 0 & 0 & 0 & 0 & 1 & 1 & 1 & 1 \\
-2 & -1 & 0 & 0 & 0 & -1 & 0 & -1 & 0 & -1 & 0 \\
1 & 0 & 0 & 0 & 1 & 0 & 1 & 0 & 1 & 0 & 0 \\
1 & 1 & 0 & 0 & 1 & 1 & 1 & 1 & 1 & 1 & 1
\end{bmatrix}
\]

Using the identification of the monomials with tuples, it was possible to compute \( H_5(\phi) \). However, the sign of this function remains unclear since it has coefficients with different signs.

We consider next the reduced network obtained by first removing all the reverse reactions and then the intermediates \( S_1F_1 \) and \( S_2F_2 \). When removing these intermediates the reactions \( S_1 + F_1 \longrightarrow S_1F_1 \longrightarrow S_0 + F_1 \) and \( S_2 + F_2 \longrightarrow S_2F_2 \longrightarrow S_1 + F_2 \) become \( S_1 + F_1 \longrightarrow S_0 + F_1 \) and \( S_2 + F_2 \longrightarrow S_1 + F_2 \) respectively. The reduced network is

\[
\begin{align*}
S_0 + E \xrightarrow{\tau_1} S_0E \xrightarrow{\tau_2} S_1 + E \\
S_1 + F_1 \xrightarrow{\tau_4} S_0 + F_1 \\
S_1 + E \xrightarrow{\tau_3} S_1E \xrightarrow{\tau_4} S_2 + E \\
S_2 + F_2 \xrightarrow{\tau_4} S_1 + F_2.
\end{align*}
\]
The species are now renamed as $S_0 = X_1, S_1 = X_2, S_2 = X_3, E = X_4, F_1 = X_5, F_2 = X_6, S_0E = X_7, S_1E = X_8$. The polynomial $q_z$ associated with this network has degree 4 and, when computing the Hurwitz determinants we have that $H_1, H_2$ and $H_3$ are positive. However the sign of $H_4$ is unclear even after evaluating at a positive parametrization of the steady state variety $\phi$. In this situation we explore the possibility of applying Theorem 1 to deduce bistability. The conservation laws of the system are

$$x_1 + x_2 + x_3 + x_7 + x_8 = T_1, \quad x_4 + x_7 + x_8 = T_2, \quad x_5 = T_3 \quad \text{and} \quad x_6 = T_4.$$ 

Taking the indices $i_1, i_2, i_3, i_4$ as 1, 4, 5, 6 respectively, we construct $F_T$ as in Eq. (3). The solutions of $F_T, \ell = 0$ for $\ell \neq 1$ are written in terms of $z = x_2$ as

$$\varphi(z) = \left( \frac{\tau_2 \tau_3 T_3 (\tau_2 z + \tau_4) z}{\tau_1 \tau_4 (\tau_2 T_2 - \tau_4 T_3) z}, \frac{\tau_2 (\tau_4 (\tau_2 T_2 - \tau_4 T_3) z)}{\tau_2 (\tau_2 z + \tau_4)}, \frac{\tau_1 (\tau_2 T_2 - \tau_4 T_3) z}{\tau_2 (\tau_2 z + \tau_4)}, \frac{\tau_1 \tau_2 T_4 z}{\tau_2 (\tau_4 z + \tau_4)} \right),$$

for $z \in \mathcal{E}$, where $\mathcal{E} = \left( 0, \frac{T_2 \tau_3}{T_3 \tau_4} \right)$.

Note that $\varphi_2(z) = z$ and $\varphi_2'(z) = 1 \neq 0$. This means that the positive steady states in the stoichiometric compatibility class defined by $T$ are in one to one correspondence with the positive roots of $F_{T,1}(\varphi(z))$ in $\mathcal{E}$. This rational function, presented below, has as numerator a polynomial of degree 3.

$$F_{T,1}(\varphi(z)) = \frac{1}{T_4 T_1 T_2 T_3 T_5 (T_1 T_2 T_3 - T_2 T_4) (\tau_1 z + \tau_4)} \left( -T_3 \tau_3 T_5 (T_3 T_1 T_2 \tau_2 \tau_3 - T_4 T_1 T_3 T_5 T_6 + T_1 T_2 \tau_4 \tau_5 T_6) z^3 - \tau_4 (T_1 T_2 T_3 T_6 (T_1 T_2 T_3 T_5 T_6 - T_2 T_4 T_1 T_3 T_5 T_6)) z^2 - \tau_4 T_2 (T_1 T_2 T_3 T_6 (T_1 T_2 T_3 T_5 T_6 - T_2 T_4 T_1 T_3 T_5 T_6)) z - \tau_4 T_2 T_3 T_4 T_5 T_6 (T_1 T_2 T_3 T_5 T_6 - T_2 T_4 T_1 T_3 T_5 T_6) \right).$$

We have already shown that the second hypothesis of Theorem 1 holds. For the first hypothesis, a straightforward computation shows that

$$\det(J_{F_T}(\varphi(z)), T_4) = \tau_1 \tau_4 T_6 T_4 (\tau_5 T_3 z - \tau_2 T_2),$$

which is negative for every $z \in \mathcal{E}$. We further have $s = 4$, $i = 2$, $j = 1$, and the independent term of the numerator of $F_{T,1}(\varphi(z))$ is negative, meaning that $(F_{T,j} \circ \varphi)'(z_1) > 0$. This gives that the sign of Eq. (11) is $(-1)^{4+1+2}(+1)(-1) = 1$ positive. Using Theorem 1 we conclude that for every set of parameters such that $F_{T,1}(\varphi(z))$ has three roots $z_1 < z_2 < z_3$ in $\mathcal{E}$, the steady states $\varphi(z_1), \varphi(z_2)$ are exponentially stable and $\varphi(z_2)$ is unstable.

All that is left is to show that the reduced network admits three positive steady states in some stoichiometric compatibility class for some choice of $\tau$, or what is the same, that $F_{T,1}(\varphi(z))$ admits three roots in $\mathcal{E}$. We consider $\det(J_{F_T}(\phi))$ for a parametrization $\phi$ of the steady states:

$$\phi(x_3, x_4, x_5, x_6) = \left( \frac{\tau_7 x_2 x_3 x_4 x_5 x_6}{\tau_3 x_3 x_4}, \frac{\tau_6 x_3 x_6}{\tau_3 x_3}, \frac{\tau_7 x_2 x_3 x_4 x_5 x_6}{\tau_3 x_3}, \frac{\tau_6 x_3 x_6}{\tau_3 x_3} \right) \quad \text{and} \quad \det(J_{F_T}(\phi)) = \tau_1 \tau_4^2 \tau_6 \tau_3 x_2 x_3 x_4 x_5 x_6^2 + \frac{\tau_2 \tau_7 \tau_6^2 x_2 x_3 x_4 x_5 x_6^2}{\tau_3 x_3} + \frac{\tau_4 \tau_7 \tau_6^2 x_2 x_3 x_4 x_5 x_6^2}{\tau_3 x_3} + \frac{\tau_1 \tau_4 \tau_6^2 x_2 x_3 x_4 x_5 x_6^2}{\tau_3 x_3} + \frac{\tau_1 \tau_4 \tau_6^2 x_2 x_3 x_4 x_5 x_6^2}{\tau_3 x_3} + \frac{\tau_1 \tau_4 \tau_6^2 x_2 x_3 x_4 x_5 x_6^2}{\tau_3 x_3} + \frac{\tau_1 \tau_4 \tau_6^2 x_2 x_3 x_4 x_5 x_6^2}{\tau_3 x_3} + \frac{\tau_1 \tau_4 \tau_6^2 x_2 x_3 x_4 x_5 x_6^2}{\tau_3 x_3}.$$ 

By letting $\tau_i = 1$, for $i = 1, \ldots, 6$ and $x_3 = 100, x_4 = 10, x_5 = 10, x_6 = 1$, $\det(J_{F_T}(\phi)) = -280$, which is negative. Hence applying the method in [14], we conclude that the stoichiometric compatibility class containing $\phi(100, 10, 10, 1)$ has more than one positive steady state. Specifically, this class corresponds to $T_1 = 320, T_2 = 210, T_3 = 10, T_4 = 1$. Either by solving the steady state equations or finding the roots of $F_{T,1}(\varphi(z))$ for this choice of parameters, we confirm that the system has three positive steady states.

Therefore, the reduced network is bistable for all choice of parameter values for which there are three positive steady states, and the original network admits bistability in some region of the parameter space.
Network (b). This network is the combination of two one-site modification cycles in a cascade, where the same phosphatase F acts in both layers.

\[
\begin{align*}
S_0 + E & \xrightarrow{\kappa_1} S_0 E \xrightarrow{\kappa_2} S_1 + E & \quad S_1 + F & \xrightarrow{\kappa_4} S_1 F \xrightarrow{\kappa_5} S_0 + F \\
P_0 + S_1 & \xrightarrow{\kappa_7 \kappa_8} P_0 S_1 \xrightarrow{\kappa_9} P_1 + S_1 & \quad P_1 + F & \xrightarrow{\kappa_{10} \kappa_{11}} P_1 F \xrightarrow{\kappa_{12}} P_0 + F.
\end{align*}
\]

We rename the species as \(E = X_1, F = X_2, S_0 = X_3, S_1 = X_4, P_0 = X_5, P_1 = X_6, S_0 E = X_7, S_1 F = X_8, P_0 S_1 = X_9, P_1 F = X_{10} \). For this network the polynomial \(q_4 \) has degree 6; and after some computations it was possible to prove that \(H_1, H_2, H_3 \) are positive polynomials. However, the sign of \(H_4 \) is unclear and the direct computation of \(H_4(\phi) \) was not feasible as the memory in a regular PC was not enough. The positive steady state variety has a monomial parametrization \(\phi \). We use the identification of monomials with tuples, to compute and analyze \(H_4(\phi) \) more efficiently. With the notation in Section 5, \(\phi \) corresponds to

\[
\beta = \left( \frac{k_4 k_6 k_{10} k_{12} (k_2 + k_3)(k_8 + k_9)}{k_1 k_3 k_7 k_9 (k_5 k_{11} + k_5 k_{12} + k_6 k_{11} + k_6 k_{12})}, 1, 1, (k_8 + k_9) k_{10} k_{12}, 1, 1, \right) \frac{k_4 k_6 k_{12} (k_8 + k_9)}{k_1 k_3 k_7 k_9 (k_5 k_{11} + k_5 k_{12} + k_6 k_{11} + k_6 k_{12})} \frac{k_4 k_6 k_{12} (k_8 + k_9)}{k_1 k_3 k_7 k_9 (k_5 k_{11} + k_5 k_{12} + k_6 k_{11} + k_6 k_{12})} \frac{k_{10} k_{12}}{k_9 (k_{11} + k_{12})} \frac{k_{10}}{k_{11} + k_{12}}.
\]

\[
B = \begin{bmatrix}
2 & 1 & 0 & 2 & 0 & 0 & 2 & 1 & 1 \\
-1 & 1 & 0 & 1 & 0 & 0 & 0 & 0 & 0 \\
-1 & 0 & 0 & -1 & 1 & 0 & 1 & -1 & 0 \\
1 & 0 & 0 & 1 & 0 & 1 & 1 & 1 & 1
\end{bmatrix}
\]

and \(\xi = (x_2, x_3, x_5, x_6)\).

Using this identification it was possible to compute \(H_4(\phi)\). However, its sign was still unclear as we encountered both positive and negative coefficients.

We then proceeded to reduce the network by removing all reverse reactions and the intermediates \(S_0 E\) and \(S_1 F\). That is, the reactions \(S_0 + E \rightarrow S_0 E \rightarrow S_1 + E\) and \(S_1 F \rightarrow S_0 + F\) are transformed into \(S_0 + E \rightarrow S_1 + E\) and \(S_1 + F \rightarrow S_0 + F\) respectively. We are left with the following reduced network

\[
\begin{align*}
S_0 + E & \xrightarrow{\tau_1} S_1 + E & \quad S_1 + F & \xrightarrow{\tau_2} S_0 + F \\
P_0 + S_1 & \xrightarrow{\tau_3} P_0 S_1 \xrightarrow{\tau_4} P_1 + S_1 & \quad P_1 + F & \xrightarrow{\tau_5} P_1 F \xrightarrow{\tau_6} P_0 + F.
\end{align*}
\]

In this network we rename the species as \(E = X_1, F = X_2, S_0 = X_3, S_1 = X_4, P_0 = X_5, P_1 = X_6, P_0 S_1 = X_9, P_1 F = X_{10} \). The polynomial \(q_4 \) has degree 4 and, after computing the Hurwitz determinants, we have that \(H_1, H_2, H_3 \) are positive. Therefore, the stability of the positive steady states depends on the sign of \(H_4(\phi)\).

The conservation laws of the system are

\[
x_1 = T_1, \quad x_5 + x_6 + x_9 + x_{10} = T_2, \quad x_3 + x_4 + x_9 = T_3, \quad x_2 + x_{10} = T_4.
\]

Taking the indices \(i_1 = 1, i_2 = 2, i_3 = 3, i_4 = 5\) we define \(F_{T, \ell} \) as in Eq. (3). Furthermore, the solutions of \(F_{T, \ell} = 0\) for \(\ell \neq 5\) can be positively parametrized in terms of \(z = x_6\) as

\[
\varphi(z) = \left( T_1, \frac{\tau_4 T_4}{\tau_5 + \tau_6}, \frac{T_4}{\tau_6} T_1 (T_3 T_4 - T_4 T_6) \tau_5 + T_3 T_4 T_6, \frac{T_5 T_3 (T_3 T_4 - T_4 T_6) \tau_5 + T_3 T_4 T_6}{T_4 (T_3 T_4 + T_3 T_6 - T_4 T_6)} \right).
\]

for \(z \in \mathcal{E}\), where \(\mathcal{E} = \mathbb{R}_{>0}\) if \(T_3 T_4 - T_4 T_6 > 0\) or \(\mathcal{E} = \left(0, \frac{T_3 T_4}{T_4 (T_3 T_4 - T_4 T_6)}\right)\) if \(T_3 T_4 - T_4 T_6 \leq 0\). Note that the positive steady states in the stoichiometric compatibility class defined by \(T\) are in one to one correspondence with the zeros of \(F_{T,5}(\varphi(z))\), below, contained in \(\mathcal{E}\).

\[
F_{T,5}(\varphi(z)) = \frac{1}{T_1 T_3 T_4 T_6 (T_3 T_4 - T_4 T_6) (T_3 T_4 + T_3 T_6 - T_4 T_6)} \left[ T_1 T_3 T_4 T_6 (T_3 T_4 - T_4 T_6) z^3 - T_1 T_3 T_4 T_6 (T_3 T_4 + T_3 T_6 - T_4 T_6) z^2 + T_1 T_3 T_4 T_6 (T_3 T_4 + T_3 T_6 - T_4 T_6) z - T_1 T_3 T_4 T_6 (T_3 T_4 + T_3 T_6 - T_4 T_6)\right].
\]

The numerator of this univariate rational function has degree 3 and the denominator is positive in \(\mathcal{E}\). Additionally, \(\varphi_6(z) = z\) and \(\varphi_6'(z) = 1 \neq 0\). With this parametrization where \(i = 6, j = 5\),
we have all the elements required in the statement of Theorem 1. We also know from the analysis of the Hurwitz determinants that the second hypothesis of Theorem 1 holds. The first hypothesis of the theorem also holds as
\[ \det(J_{\mathcal{F}_T}(\varphi(z)))_{i,j} = -\tau_j T_1 T_4 (T_3 T_4 - T_4 T_6) \tau_5 z + T_3 T_1 T_6 \]
is negative for every \( z \in \mathcal{E} \). Using Theorem 1 and the fact that the independent term of \( \mathcal{F}_{T,5}(\varphi(z)) \) is negative, we conclude that for every set of parameters such that \( \mathcal{F}_{T,5}(\varphi(z)) \) has 3 positive roots \( z_1 < z_2 < z_3 \) in \( \mathcal{E} \), the sign of Eq. (11) is \((-1)^6+5+4+1(-1) = 1 \) and thus \( \varphi(z_1), \varphi(z_3) \) are exponentially stable and \( \varphi(z_2) \) is unstable.

It remains to see that there is a set of parameters such that the network admits three positive steady states. We find \( \det(J_{\mathcal{F}_T}(\phi)) \) for a parametrization \( \phi \):
\[
\phi(x_2, x_3, x_5, x_6) = \left( \frac{\tau_2 \tau_5 x_2^2 x_6}{\tau_1 \tau_5 x_5}, x_2, x_3, \frac{\tau_2 \tau_5 x_2 x_6}{\tau_1 \tau_5}, x_6, \frac{\tau_2 \tau_5 x_2 x_6}{\tau_4}, \frac{\tau_2 \tau_5 x_2 x_6}{\tau_6} \right)
\]
and
\[
\det(J_{\mathcal{F}_T}(\phi)) = \frac{1}{x_1 x_2^2} \left( \tau_2 \tau_5 x_2^2 (\tau_1 \tau_5 x_5 x_2 x_3 x_5 x_6 - \tau_3 \tau_5 \tau_3 x_3 x_5^2 x_6 + \tau_3 \tau_3 x_3 x_5 x_6) + \tau_3 \tau_5 \tau_6 x_2 x_3 x_5 x_6 + \tau_3 \tau_6 x_2 x_3 x_5 x_6 + \tau_3 \tau_6 x_2 x_3 x_6 + \tau_3 \tau_6 x_2 x_5 + \tau_3 \tau_6 x_3 x_5 x_6 + \tau_4 \tau_5 \tau_6 x_2 x_3 x_5 x_6 + \tau_4 \tau_5 \tau_6 x_2 x_3 x_6 + \tau_4 \tau_6 \right).
\]
Taking \( \tau_i = 1 \) for \( i = 1, \ldots, 6 \) and \( x_2 = 1, x_3 = 10, x_5 = 20, x_6 = 10 \) this determinant is -4500. By [14], the stoichiometric compatibility class containing \( \phi(1, 10, 20, 10) \) has more than one positive steady state. The total amounts defining it are \( T_1 = \frac{1}{2}, T_2 = 50, T_3 = \frac{4}{1}, T_4 = 11 \). Using these parameters and solving either \( \mathcal{F}_T(x) = 0 \) or finding the roots of \( \mathcal{F}_{T,5}(\phi) \) we verify that this stoichiometric compatibility class has in fact three positive steady states.

We conclude that the reduced network is bistable for every set of parameters for which there are three positive steady states, and the original network admits bistability in some region of the parameter space.

**Network (c).** This network is the combination of two one-site modification cycles in a cascade, where the same kinase \( E \) acts in both layers:

\[
\begin{align*}
S_0 + E & \xrightarrow{\kappa_1} S_0 E \xrightarrow{\kappa_3} S_1 + E \\
P_0 + S_1 & \xrightarrow{\kappa_2} P_0 S_1 \xrightarrow{\kappa_9} P_1 + S_1 \\
P_1 + F_2 & \xrightarrow{\kappa_10} P_1 F_2 \xrightarrow{\kappa_12} P_0 + F_2.
\end{align*}
\]

The species are renamed as \( S_0 = X_1, S_1 = X_2, P_0 = X_3, P_1 = X_4, E = X_5, F_1 = X_6, F_2 = X_7, S_0 E = X_8, S_1 F_1 = X_9, P_0 S_1 = X_{10}, P_1 F_2 = X_{11}, P_0 E = X_{12} \). For this network, the polynomial \( q_5 \) has degree 7 and the determinants \( H_1, H_2, H_3 \) are positive polynomials. However, the computations of the other determinants was not possible as there was not enough memory to store the computations in a regular PC. In this case, we could not parametrize the positive steady state variety by monomials; therefore, it is not possible to use the identification between monomials and tuples to analyze the sign of the remaining determinants.

We proceed directly to reduce the network by removing all the reverse reactions first, and then the intermediates \( S_0 E, P_0 S_1 \) and \( P_1 F_2 \). That is, the reactions \( S_0 + E \rightarrow S_0 E \rightarrow S_1 + E \), \( P_0 + S_1 \rightarrow P_0 S_1 \rightarrow P_1 + S_1 \) and \( P_1 + F_2 \rightarrow P_1 F_2 \rightarrow P_0 + F_2 \) are transformed into \( S_0 + E \rightarrow S_1 + E \), \( P_0 + S_1 \rightarrow P_1 + S_1 \) and \( P_1 + F_2 \rightarrow P_0 + F_2 \) respectively. We are left with the following network

\[
\begin{align*}
S_0 + E & \xrightarrow{\tau_1} S_1 + E \\
P_0 + S_1 & \xrightarrow{\tau_4} P_1 + S_1 \\
P_0 + E & \xrightarrow{\tau_6} P_0 E \xrightarrow{\tau_7} P_1 + E \\
P_1 + F_2 & \xrightarrow{\tau_12} P_0 + F_2.
\end{align*}
\]

The species are renamed as \( S_0 = X_1, S_1 = X_2, P_0 = X_3, P_1 = X_4, E = X_5, F_1 = X_6, F_2 = X_7, S_1 F_1 = X_9, P_0 E = X_{12} \). The polynomial \( q_5 \) associated with this network has degree 4 and \( H_1, H_2, H_3 \) are positive after evaluating in the following positive parametrization \( \phi \) of the steady state variety:
However, the sign of $H_4$ is unclear. The conservation laws of the system are

$$x_1 + x_2 + x_9 = T_1, \quad x_3 + x_4 + x_{12} = T_2, \quad x_5 + x_{12} = T_3, \quad x_6 + x_9 = T_4, \quad x_7 = T_5.$$  

Taking $i_1 = 1, i_2 = 3, i_3 = 5, i_4 = 6, i_5 = 7$ we define $F_T$ as in Eq. (3). Additionally, the solutions of $F_{\ell T}(x) = 0$ for $\ell \neq 6$ can be parametrized in terms of $z = x_3$ as

$$\varphi(z) = \left( \frac{b_1(z) \tau_1}{\tau_3 \tau_3 \tau_3 + \tau_3 \tau_1 \tau_7 + \tau_1 \tau_7}, \frac{b_2(z)}{\tau_3 \tau_3 \tau_3 + \tau_1 \tau_7 + \tau_7}, \frac{(-76z^2 + (T_2 \tau_1 - T_3 \tau_1 - \tau_7)z + T_2 \tau_1)}{\tau_6 \tau_2 + \tau_7} \right).$$

where

$$b_1(z) = (T_1 \tau_4 + T_3 \tau_5) \tau_6 z^2 + (-T_2 T_5 \tau_5 \tau_6 + T_3 T_5 \tau_5 \tau_6 + T_1 \tau_4 \tau_7 + T_3 \tau_5 \tau_7 + T_5 \tau_7) z - T_2 T_5 \tau_5 \tau_7,$$

$$b_2(z) = -T_3 \tau_5 \tau_6 z^2 + (T_2 T_5 \tau_5 \tau_6 - T_3 T_5 \tau_5 \tau_7 - T_5 \tau_7) z - T_2 T_5 \tau_5 \tau_7.$$  

The parametrization is positive if and only if $b_1(z), b_2(z) > 0$. This happens for $z \in \mathcal{E}$, where $\mathcal{E} = (\beta_1, \beta_2)$ with $\beta_1$ and $\beta_2$ the (only) positive roots of the polynomials $b_1$ and $b_2$ respectively.

The steady states in each stoichiometric compatibility class are in one to one correspondence with the roots of $F_{T,6}(\varphi(z))$ in $\mathcal{E}$. The numerator of this function is a polynomial of degree 5 and the denominator is positive in $\mathcal{E}$. Additionally, $\varphi_3(z) = z$ and $\varphi_6'(z) = 1 \neq 0$. With this parametrization and taking $i = 3, j = 6, s = 4$, we have all the elements in the statement of Theorem 1. The first three Hurwitz determinants are positive and thus, the second hypothesis of the theorem holds. The first hypothesis holds as

$$\det(J_F(\varphi(z)),_{T,i}) = \frac{-\tau_2(\tau_3 \tau_6 z + T_3 \tau_1 \tau_7 + \tau_3 \tau_7) b_2(z)}{\tau_6 \tau_2 + \tau_7},$$

is negative for all $z \in \mathcal{E}$. We need to decide the sign of $(F_{T,j} \circ \varphi)'(z_1)$ at the first root of the numerator of $F_{T,j} \circ \varphi$. Indirect evaluation of $F_{T,j} \circ \varphi$ at $\beta_1$, by isolating $T_2$ from $b_2(z) = 0$ and substitution into $F_{T,j} \circ \varphi$, shows that $F_{T,j} \circ \varphi$ is negative at $\beta_1$. Hence the derivative at $z_1$ is positive. By Theorem 1 we conclude that for every set of parameters such that $F_{T,6}(\varphi(z))$ has more than two positive roots $z_1, z_2, \ldots$, the sign of Eq. (11) is $(-3)^3(-1)^4(1)(-1) = 1$ positive. Therefore, the steady states $\varphi(z_1), \varphi(z_2), \ldots$ are exponentially stable and $\varphi(z_2), \ldots$ are unstable.

It remains to see that there is a set of parameters such that the stoichiometric compatibility class in fact contains more than two positive steady states. We find $\det(J_F(\varphi))$, with $\varphi$ as above:

$$\det(J_F(\varphi)) = -\tau_2(\tau_3 \tau_6 z + T_3 \tau_1 \tau_7 + \tau_3 \tau_7) b_2(z).$$

Taking $\tau_1 = 1$ for $i = 1, \ldots, 7$ and $x_1 = 40, x_2 = 10, x_3 = 1, x_6 = 1, x_7 = 1$, this determinant is $-32,000$. By 14, it follows that the stoichiometric compatibility class containing $\phi(x)$ has more than one positive steady state. The total amounts defining the stoichiometric compatibility class are $T_1 = 60, T_2 = \frac{23}{T}, T_3 = \frac{1}{T}, T_4 = 11, T_5 = 1$. Using these parameters and solving either $F_T(x) = 0$ or $F_{T,6}(\varphi(z)) = 0$ we verify that this stoichiometric compatibility class has three positive steady states as desired.

The reduced network is thus bistable whenever it has three positive steady states, and the original network admits bistability in some parameter region.
The species are renamed as \(E\) and \(X\). In this case, the positive steady state variety can be parametrized by monomials \(\phi\) can be parametrized in terms of every positive steady state. Regarding the determinants \(H\), the two sites of its phosphorylation \(S\) denotes the network. The solutions of \(H\) prove that the sign of \(H\) is unclear and the analysis of the sign of \(H\) was stopped as the computations could not be stored in a regular PC. In this case, the positive steady state variety can be parametrized by monomials \(\phi\), which, with the notation in Section 3 corresponds to

\[
\beta = \left( \frac{(k_2 + k_3)k_4k_6k_{10}k_{12}(k_5 + k_9)}{k_1k_3k_5k_9(k_5 + k_6)(k_{11} + k_{12})}, \frac{(k_8 + k_9)k_{10}k_{12}}{k_3k_5k_6k_{11} + k_{12}} \right),
\]

\(B = \begin{bmatrix} 1 & 0 & -2 & -1 & 0 & -1 & 0 & 0 & -1 \\ 0 & 1 & 2 & 1 & 0 & 2 & 1 & 1 & 2 \\ 0 & 0 & 1 & 1 & 1 & 1 & 1 & 1 & 1 \end{bmatrix}\) and \(\xi = (x_1, x_2, x_3)\).

Using tuples, it was possible to compute \(H_4(\phi)\) and \(H_5(\phi)\) and, after studying their sign, we found that \(H_4(\phi)\) is a positive polynomial, but the sign of \(H_5(\phi)\) was not clear.

We then proceed to reduce the network by removing all the reverse reactions and the intermediates \(S\). That is, the reactions \(S_1 + E \rightarrow S_1E \rightarrow S_2 + E, S_2 + F \rightarrow S_2F \rightarrow S_1 + F\) and \(S_1 + F \rightarrow S_1 + F \rightarrow S_0 + F\). The reduced network is

\[
S_0 + E \rightarrow ES_0 \rightarrow S_1 + E \rightarrow S_2 + F \rightarrow S_0 + F.
\]

We rename the species as \(X_1, F = X_2, S_0 = X_3, S_1 = X_4, S_2 = X_5, S_0E = X_6\). The polynomial \(q_x\) has degree 3 and, after computing the Hurwitz determinants, it was possible to prove that \(H_1\) and \(H_2\) are polynomials with positive coefficients and, thus positive for every positive steady state. However, the sign of \(H_3\) was unclear even after evaluating at the following positive parametrization \(\phi\):

\[
\phi(x_1, x_2, x_3) = \begin{bmatrix} x_1, x_2, x_3, \frac{T_1x_1x_3}{T_2}, \frac{T_3x_1x_3}{T_5x_2}, \frac{T_1x_1x_3}{T_2} \end{bmatrix}.
\]

The conservation laws of the system are \(x_1 + x_5 = T_1, x_2 = T_2\) and \(x_3 + x_4 + x_5 = x_6 = T_3\). Taking \(i_1 = 1, i_2 = 2, i_3 = 3\) we define \(F_T\) as in Eq. (3). The solutions of \(F_{T,\ell}(x) = 0\) for \(\ell \neq 1\) can be parametrized in terms of \(z = x_1\) as

\[
\varphi(z) = \begin{bmatrix} z, T_2, \frac{T_2T_3T_4T_5}{T_1T_2T_3T_4T_5}, \frac{T_2T_3T_4T_5}{T_1T_2T_3T_4T_5}, \frac{T_2T_3T_4T_5}{T_1T_2T_3T_4T_5}, \frac{T_2T_3T_4T_5}{T_1T_2T_3T_4T_5} \end{bmatrix}.
\]

This parametrization is positive for every \(z \in \mathbb{R}_{>0}\) and the positive steady states in the stoichiometric compatibility class are in one to one correspondence with the positive roots of \(F_{T,1}(\varphi(z))\). This is a rational function whose numerator is a polynomial of degree 3 and positive denominator:

\[
F_{T,1}(\varphi(z)) = \frac{1}{T_1T_2T_3T_4T_5 + T_2T_3T_4T_5 + T_3T_4T_5 + T_4T_5 + T_5} \left[ T_1T_2T_3T_4T_5 + T_2T_3T_4T_5 + T_3T_4T_5 + T_4T_5 + T_5 \right] + \left( -T_1T_2T_3T_4T_5 + T_2T_3T_4T_5 + T_3T_4T_5 + T_4T_5 + T_5 \right).
\]

Therefore, there are at most three positive steady states in each stoichiometric compatibility class. Additionally \(\varphi_1(z) = z\) and \(\varphi_2(z) = 1 \neq 0\). With this parametrization, where, \(i = j = 1\) and \(s = 3\), we have all the elements in the statement of Theorem (1) The second hypothesis of the theorem holds, and the first hypothesis also does as

\[
\det(J_F(\varphi(z)))_{j,l} = -T_2T_1T_4T_5z - T_1T_4T_5z^2 - T_2T_1T_2T_4z - T_2T_2T_4z.
\]
is negative for every $z \in \mathbb{R}_{>0}$. We are in the setting of Theorem 1 and we conclude that for every set of parameters such that $F_{T,1}(\varphi(z))$ has three positive roots $z_1 < z_2 < z_3$, the sign of $(F_{T,j} \circ \varphi)'(z_1)$ is positive as the independent term of $(F_{T,j} \circ \varphi)(z)$ is negative. Furthermore, the sign of Eq. (11) is equal to $(-1)^{1+1+3}(1)(-1) = 1$, which is positive. This implies that the steady states $\varphi(z_1), \varphi(z_3)$ are exponentially stable and $\varphi(z_2)$ is unstable.

We now verify that there exists a set of parameters such that the network has three positive steady states. As above, we have

$$\det(J_{P'}(\phi)) = -\tau_7^2 \tau_3 x_1^2 x_3 + \tau_1 \tau_4 \tau_5 x_1 x_3^2 + \tau_1 \tau_4 \tau_5 x_2 x_3 + \tau_1 \tau_2 \tau_3 x_1^2 + \tau_1 \tau_2 \tau_4 x_1 x_2 + \tau_2 \tau_4 \tau_5 x_2^2.$$  

Taking $\tau_1 = 1$ for $i = 1, \ldots, 5$ and $x_1 = 5, x_2 = 1, x_3 = 5$, this determinant is equal to -84. By [12], the stoichiometric compatibility class containing $\phi(x)$ has more than one positive steady state. The total amounts defining it are $T_1 = 30, T_2 = 1, T_3 = 180$. Using these parameters and solving either $F_T(x) = 0$ or $F_{T,1}(\varphi(z)) = 0$ we prove that there are three positive steady states in his stoichiometric compatibility class, as desired.

The reduced network is bistable whenever it has three positive steady states, and hence the original network admits bistability in some parameter region.

**Network (e).** In this network two substrates $S_0$ and $P_0$ are phosphorylated by the same kinase $E$, and dephosphorylated by the same phosphatase $F$.

$$S_0 + E \xrightleftharpoons{\kappa_1}{\kappa_2} S_0 E \xrightarrow{\kappa_3} S_1 + E \quad S_1 + F \xrightleftharpoons{\kappa_4}{\kappa_5} S_1 F \xrightarrow{\kappa_6} S_0 + F$$

$$P_0 + E \xrightleftharpoons{\kappa_7}{\kappa_8} P_0 E \xrightarrow{\kappa_9} P_1 + E \quad P_1 + F \xrightleftharpoons{\kappa_{10}}{\kappa_{11}} P_1 F \xrightarrow{\kappa_{12}} P_0 + F.$$  

We rename the species as $E = X_1, F = X_2, S_0 = X_3, S_1 = X_4, P_0 = X_5, P_1 = X_6, S_0 E = X_7, S_1 F = X_8, P_0 E = X_9, P_1 F = X_{10}$. The polynomial $q_x$ has degree 6, and the first three Hurwitz determinants are positive for every positive steady state. The sign of $H_4$ is unclear, and the analysis of the sign of $H_5$ was interrupted due to memory problems.

The positive steady state variety admits a parametrization $\phi$ by monomials, which in the notation of Section 5, corresponds to

$$\beta = \left(1, 1, \frac{\kappa_3 \kappa_6 (\kappa_2 + \kappa_3)}{\kappa_1 \kappa_3 (\kappa_5 + \kappa_6)}, 1, \frac{\kappa_4 \kappa_6 (\kappa_2 + \kappa_3)}{(\kappa_1 + \kappa_2) \kappa_7 \kappa_9}, 1, \frac{\kappa_4 \kappa_6 \kappa_8 \kappa_9 (\kappa_2 + \kappa_3)}{\kappa_3 (\kappa_5 + \kappa_6)}, \frac{\kappa_4 \kappa_6 \kappa_8 \kappa_9 (\kappa_1 + \kappa_2)}{\kappa_5 \kappa_6 \kappa_9}, \frac{\kappa_4 \kappa_6 \kappa_8 \kappa_9 (\kappa_1 + \kappa_2)}{\kappa_5 \kappa_6 \kappa_9}, \frac{\kappa_4 \kappa_6 \kappa_8 \kappa_9 (\kappa_1 + \kappa_2)}{\kappa_5 \kappa_6 \kappa_9}ight),$$

$$B = \begin{bmatrix}
1 & 0 & -1 & 0 & -1 & 0 & 0 & 0 & 0 & 0 \\
0 & 1 & 1 & 0 & 1 & 0 & 1 & 1 & 1 & 1 \\
0 & 0 & 1 & 1 & 0 & 0 & 1 & 0 & 1 & 0 \\
0 & 0 & 0 & 0 & 1 & 1 & 0 & 1 & 0 & 1 \\
\end{bmatrix} \quad \text{and} \quad \xi = (x_1, x_2, x_4, x_6).$$

Using tuples, we computed $H_4(\phi)$ and $H_5(\phi)$ and, after studying the signs of their coefficients, we have that $H_4(\phi)$ is positive but the sign of $H_5(\phi)$ is still unclear.

We proceed to reduce the network by removing all the reverse reactions and the intermediates $S_0 E$ and $P_1 F$. That is, the reactions $S_0 + E \rightarrow S_0 E \rightarrow S_1 + E$ and $P_1 + F \rightarrow P_1 F \rightarrow P_0 + F$ become $S_0 + E \rightarrow S_1 + E$ and $P_1 + F \rightarrow P_0 + F$ respectively. The reduced network is

$$S_0 + E \xrightarrow{\tau_1} S_1 + E \quad S_1 + F \xrightarrow{\tau_2} S_1 F \xrightarrow{\kappa_3} S_0 + F$$

$$P_0 + E \xrightarrow{\tau_4} P_0 E \xrightarrow{\kappa_5} P_1 + E \quad P_1 + F \xrightarrow{\tau_6} P_0 + F.$$  

The species are renamed as $E = X_1, F = X_2, S_0 = X_3, S_1 = X_4, P_0 = X_5, P_1 = X_6, S_1 F = X_7, P_0 E = X_8$. The polynomial $q_x$ has degree 4. The Hurwitz determinants $H_1, H_2$ and $H_3$ are positive for every positive steady state. However, the sign of $H_4$ is unclear even after evaluating at the parametrization $\phi$ below:

$$\phi(x_1, x_2, x_4, x_6) = \left(\frac{\tau_2 x_2 x_4}{\tau_1 x_1}, \frac{\tau_6 x_2 x_6}{\tau_4 x_1}, \frac{\tau_6 x_2 x_6}{\tau_5}, \frac{\tau_7 x_2 x_4}{\tau_3}\right).$$

The conservation laws of the system are

$$x_1 + x_8 = T_1, \quad x_2 + x_9 = T_2, \quad x_3 + x_4 + x_9 = T_3 \quad \text{and} \quad x_5 + x_6 + x_8 = T_4.$$
Taking \( i_1 = 1, i_2 = 2, i_3 = 3, i_4 = 5 \) we define \( F_T(x) \) as in Eq. (3). Furthermore, the solutions of \( F_{T,\ell}(x) = 0 \) for \( \ell \neq 2 \) can be positively parametrized in terms of \( z = x_5 \) as

\[
\varphi(z) = \left( \frac{T_1 \tau_7}{\tau_4 z + \tau_5}, \frac{-(T_1 - T_3 \tau_4 \tau_5) z - T_3 \tau_5}{b_2(z)} \right),
\]

where

\[
b_1(z) := \tau_4 z^2 + ((T_1 - T_3) \tau_4 + \tau_5) z - T_4 \tau_5,
\]

\[
b_2(z) := (-(T_1 \tau_4 T_3 \tau_4 + \tau_2 \tau_3 \tau_4) z^2 + (T_1 \tau_4 T_2 \tau_4 + T_3 \tau_3 \tau_4 T_6 + T_4 \tau_4 \tau_3 \tau_4 \tau_6 - \tau_4 \tau_3 \tau_6 \tau_6 + \tau_2 \tau_3 \tau_4 \tau_6) z + T_3 \tau_4 \tau_3 \tau_6 \tau_6).
\]

Here \( \mathcal{E} = (0, \beta_1) \) where \( \beta_1 \) is the positive root of \( b_1(z) \), such that \( b_1(z) < 0 \) and \( b_2(z) > 0 \) in \( \mathcal{E} \).

The positive steady states in the stoichiometric compatibility class are in one to one correspondence with the roots of \( F_{T,2}(\varphi(z)) \). This is a rational function whose numerator is a polynomial of degree four with positive independent term and denominator positive in \( \mathcal{E} \). Additionally, \( \varphi_5(z) = z \) and \( \varphi'_5(z) = 1 \neq 0 \) and taking \( i = 5, j = 2 \) we have all the elements in the statement of Theorem 1. The second hypothesis in the theorem holds, and so does the first as

\[
\det(J_F(\varphi(z)),J_I) = -\frac{T_1 \tau_5 b_2(z)}{\tau_4 z + \tau_5}
\]

is negative for \( z \in \mathcal{E} \). The sign of \( (F_{T,2} \circ \varphi)’(z_1) \), for \( z_1 \) the first root of \( (F_{T,2} \circ \varphi)(z_1) \), is positive as the independent term of \( (F_{T,2} \circ \varphi)(z) \) is negative. Hence, the sign of Eq. (11) is \((-1)^{5+2-1}(1)(-1) = 1\), which is positive. Thus, by Theorem 1 we conclude that the steady states \( \varphi(z_1), \varphi(z_3) \) are exponentially stable and \( \varphi(z_2), \ldots \) are unstable.

To show that the network admits three positive steady states, we consider \( \det(J_F(\phi)) \):

\[
\det(J_F(\phi)) = T_1 T_2 T_3 T_6 x_1 x_2^2 + T_1 T_2 T_3 T_6 x_4 x_2^2 + T_1 T_2 T_3 T_6 x_4 x_2^2 + T_1 T_2 T_3 T_6 x_4 x_2^2 + T_1 T_2 T_3 T_6 x_4 x_2^2 + T_1 T_2 T_3 T_6 x_4 x_2^2 + T_1 T_2 T_3 T_6 x_4 x_2^2
\]

Taking \( \tau_1 = \tau_2 = \tau_4 = \tau_5 = \tau_6 = 1, \tau_2 = 2 \) and \( x_1 = x_2 = 1, x_4 = 9, x_6 = 9 \), the value of the determinant is -48. By\[1, \]the stoichiometric compatibility class containing \( \phi(1,1,9,9) \) has more than one positive steady states and, solving either \( F_T(x) = 0 \) or \( F_{T,2}(\varphi(z)) = 0 \) we verify that it has three positive steady states.

The reduced network is bistable for every set of parameters for which there are three positive steady states, and hence the original network admits bistability in some parameter region.

**Network (f).** \( E \) corresponds to a kinase that exists in two conformations: \( E_1 \) (relaxed state) and \( E_2 \) (tensed state) \[34\]. Each conformation acts as a kinase for a common substrate \( S_0 \). We denote by \( S_1 \) the phosphorylated form of the substrate. We assume that the intermediate kinase-substrate complexes, \( E_1 S_0 \) and \( E_2 S_0 \), also undergo conformational change.

\[
E_1 + S_0 \xrightarrow{\kappa_1} E_1 S_0 \xrightarrow{\kappa_3} E_1 + S_1 \quad E_2 + S_0 \xrightarrow{\kappa_4,\kappa_5} E_2 S_0 \xrightarrow{\kappa_6} E_2 + S_1
\]

\[
E_1 \xrightarrow{\kappa_8} E_2 \quad E_1 S_0 \xrightarrow{\kappa_{10},\kappa_{11}} E_2 S_0 \quad S_1 \xrightarrow{\kappa_7} S_0.
\]

The species are renamed as \( E_1 = X_1, E_2 = X_2, E_1 S_0 = X_3, E_2 S_0 = X_4, S_0 = X_5, S_1 = X_6 \). The polynomial \( q_0 \) associated with this network has degree 4. After computing and evaluating the Hurwitz determinants at a positive parametrization \( \phi \) of the positive steady state variety, the sign of \( H_3 \) and \( H_4 \) is unclear. Since the hypotheses of Theorem 1 do not hold, we reduce the network by removing all the reverse reactions (with rate constants \( \kappa_2, \kappa_5, \kappa_9, \kappa_{10} \)) and the intermediate \( E_2 S_0 \). When removing \( E_2 S_0 \), the reactions \( E_2 + S_0 \to E_2 S_0 \to E_2 + S_1 \) become \( E_2 + S_0 \to E_2 + S_1 \) and the path \( E_2 + S_0 \to E_2 S_0 \to E_1 S_0 \) is collapsed to \( E_2 + S_0 \to E_1 S_0 \). The intermediate \( E_2 S_0 \) is not of the type considered in the text, where the technical condition on the rate constants for lifting bistability holds. But the condition holds by\[53\] Prop. 5.3(iii)].
The reduced network is

$$
E_1 + S_0 \xrightarrow{\tau_1} E_1 S_0 \xrightarrow{\tau_2} E_1 + S_1 \quad E_2 + S_0 \xrightarrow{\tau_3} E_2 + S_1
\quad E_2 + S_0 \xrightarrow{\tau_6} E_1 S_0 \xrightarrow{\tau_7} E_2 \quad S_1 \xrightarrow{\tau_4} S_0.
$$

The species are renamed as $E_1 = X_1, E_2 = X_2, E_1 S_0 = X_3, S_0 = X_5, S_1 = X_6$. We have $s = 3$. For this reduced network, $q_s$ has degree 3 and the determinants $H_1, H_2$ are positive for every positive steady state. This means that the stability of steady states depends on the sign of $H_3$.

We explore the possibility of applying Theorem 1 to ensure bistability. The conservation laws of the network are $x_1 + x_2 + x_3 = T_1$ and $x_3 + x_5 + x_6 = T_2$. Taking $i_1 = 1$ and $i_2 = 3$ we define $F_T(x)$ as in Eq. (3). The solutions of $F_T(x) = 0$ for $\ell \neq 3$ can be parametrized in terms of $z = x_5$ as

$$
\varphi(z) = \left(\frac{T_1 \tau_7 \tau_6 z + T_1 \tau_2 \tau_5 z}{b(z)}, \frac{T_1 \tau_6 z (\tau_1 z + \tau_6)}{b(z)}, \frac{T_1 \tau_2 z (\tau_1 \tau_6 z + \tau_3 \tau_6 + \tau_5 \tau_6)}{\tau_4 b(z)}\right),
$$

with $b(z) = \tau_1 \tau_6 z^2 + (\tau_2 + \tau_7) \tau_6 z + \tau_2 \tau_6$, and this parametrization is positive for every $z \in E = \mathbb{R}_{>0}$. Additionally $\varphi_4(z) = z$ and $\varphi_4'(z) = 1 \neq 0$. With this parametrization, the positive steady states in one stoichiometric compatibility class are in one to one correspondence with the positive roots of $F_{T,3}(\varphi(x)) = 0$, that is a rational function whose numerator is a polynomial of degree 3 and has positive denominator:

$$
F_{T,3}(\varphi(x)) = \frac{1}{(\tau_1 \tau_6 z^2 + \tau_2 \tau_6 z + \tau_6 \tau_6 z + \tau_2 \tau_6 \tau_6 \tau_7 \tau_4)} \left(\tau_1 \tau_4 \tau_6 \tau_7 z^3 + (T_1 \tau_1 \tau_2 \tau_6 + T_1 \tau_1 \tau_4 \tau_6 - T_1 \tau_4 \tau_6 + T_2 \tau_4 \tau_6 + T_2 \tau_4 \tau_6 + T_4 \tau_4 \tau_6) z^2 + (T_1 \tau_2 \tau_4 \tau_6 + T_1 \tau_4 \tau_6 \tau_7 - T_1 \tau_4 \tau_6 \tau_7 - T_2 \tau_4 \tau_6 \tau_7 - T_2 \tau_4 \tau_6 \tau_7 - T_2 \tau_4 \tau_6 \tau_7 z - T_2 \tau_4 \tau_6 \tau_7 z\right).
$$

Here $i = 4, j = 3$. The second hypothesis of Theorem 1 holds. For the first hypothesis, we have

$$
\det(J_F(\varphi(z), I)) = \tau_4 b(z),
$$

which is positive for every $z \in E$. The sign of $(F_{T,3} \circ \varphi)'(z_1)$, for $z_1$ the first positive root of $F_{T,3}(\varphi(z))$, is positive as the independent term of $(F_{T,3} \circ \varphi)(z)$ is negative. Furthermore, the sign of Eq. (11) is $(-1)^{3+3+4}/(1)(1) = 1$ positive. By Theorem 1, the steady states $\varphi(z_1), \varphi(z_3), \ldots$ are exponentially stable and $\varphi(z_2), \ldots$ are unstable.

To verify the existence of three positive steady states, we consider $\det(J_F(\phi))$ for $\phi$ as follows:

$$
\phi(x_2, x_5) = \left(\frac{\tau_6 \tau_2 x_2 x_5}{\tau_5}, \frac{\tau_6 \tau_2 x_2 x_5 (\tau_1 x_2 + \tau_6)}{\tau_2 \tau_5}, \frac{x_2 x_5 (\tau_1 \tau_6 x_5 + \tau_3 \tau_6 + \tau_5 \tau_6)}{\tau_4 \tau_5}, x_5, \tau_2 \tau_5 x_2 + (\tau_2 + \tau_4) \tau_5 \tau_6 x_2 x_5 + (\tau_2 \tau_6 + \tau_4 \tau_6) \tau_5^2 x_2 + \tau_1 \tau_4 \tau_5 \tau_6 x_2^2 + (\tau_2 + \tau_6) \tau_4 \tau_5 \tau_6 x_5 + \tau_2 \tau_4 \tau_5^2\right).
$$

Taking $\tau_i = 1$ for $i = 1, \ldots, 6$ and $x_2 = 2, x_5 = 6$, $\det(J_F(\phi)) = -35$. By [11], the stoichiometric compatibility class containing $\phi(x)$ has more than one positive steady state. This class is defined by the total amounts $T_1 = 98, T_2 = 222$ and by solving $F_T(x) = 0$ or $F_{T,3}(\varphi(z)) = 0$, we verify that there are three positive steady states.

Hence the reduced network is bistable for every set of parameters for which there are three positive steady states, and the original network admits bistability in some parameter region.